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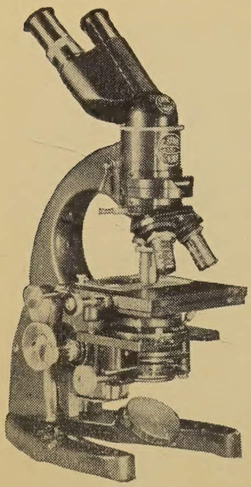

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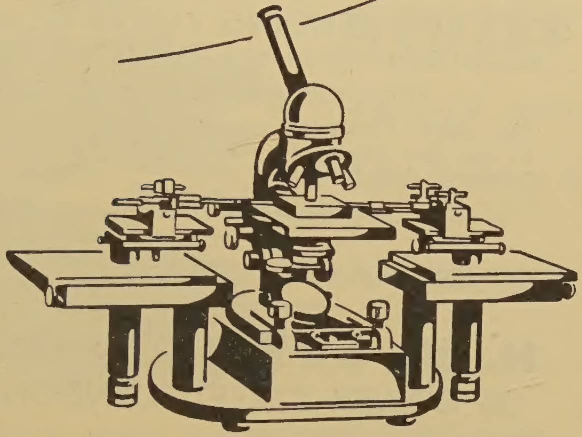

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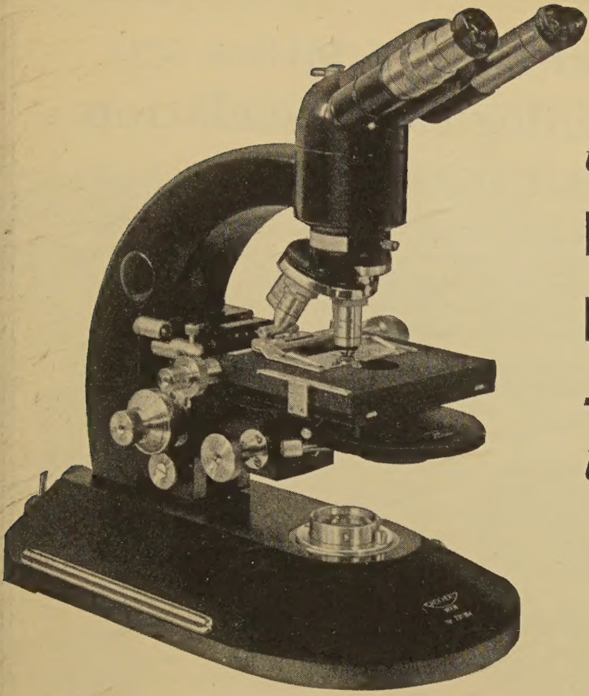

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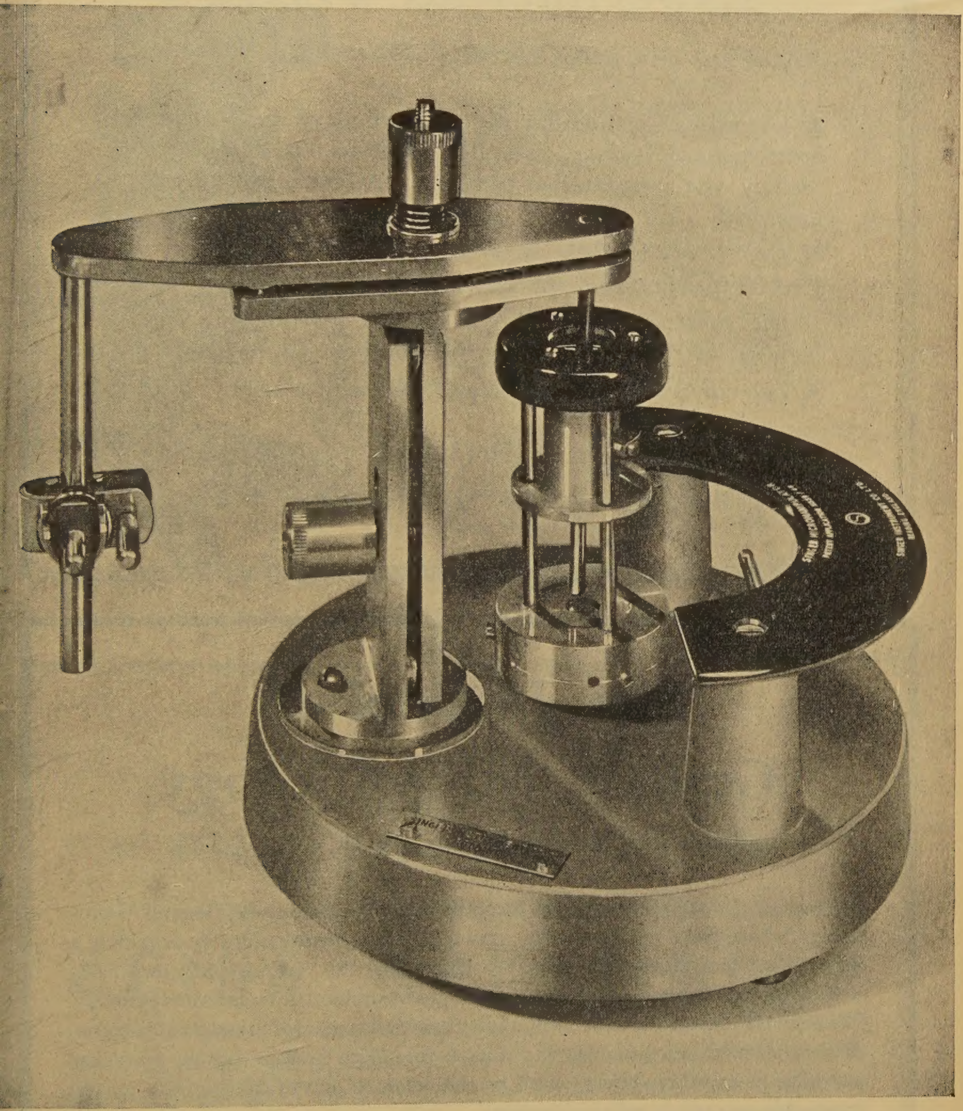

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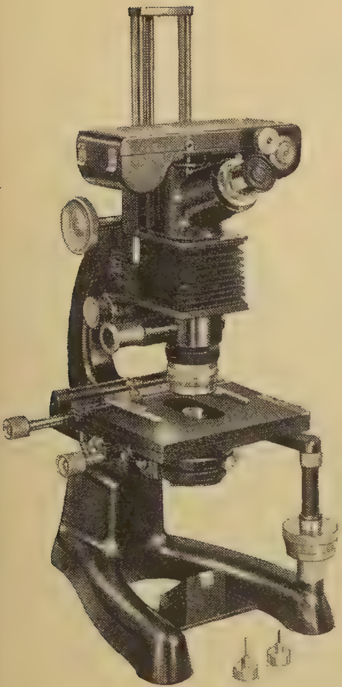

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About three-quarters of the book is concerned with birds, since the factors regulating natural populations of birds have been studied particularly. There is, however, discussion of the problem as it is found in mammals, fish, and insects. There has been more investigation in north-western Europe and North America than elsewhere and consequently examples are taken mainly from these areas, although examples from other areas have been included when relevant.

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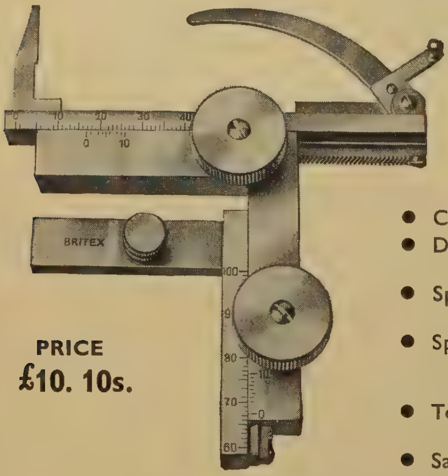

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Histochemical Methods for Protein-bound Sulphydryl and Disulphide Groups

By G. GOMORI

(*From the Department of Medicine, the University of Chicago*)

With 2 plates (figs. 1 and 2)

SUMMARY

Histochemical methods for the localization of protein-bound sulphydryl groups were compared in a critical fashion. As far as localization is concerned, several of the methods published were found to be satisfactory. However, from the point of view of approximate visual quantitation, the results are confusing. The two best methods for the histochemical detection of protein-bound sulphydryl groups are the tetrazolium method and Barrnett and Seligman's dihydroxydinaphthyldisulphide technique.

IN the course of investigations on the islets of Langerhans by the tetrazolium and the dihydroxydinaphthyldisulphide (DDD) methods it was found that the results obtained by these two techniques were not strictly identical. This discrepancy prompted a comparative study of various methods for the demonstration of protein-bound sulphydryl and disulphide groups.

Both sulphydryl and disulphide groups occur in various proteins. The former can be demonstrated directly by sulphydryl reagents while the latter must be first reduced to sulphydryls. The present paper will deal only with the demonstration of the sum of sulphydryls and disulphides for the following reason.

Sulphydryl compounds are quite sensitive to even mild oxidative agencies such as exposure to air, and are converted to disulphides. The only fixative which is claimed to preserve sulphydryls is acid alcohol (80% alcohol containing 1% trichloro-acetic acid). This mixture is a poor fixative which has a powerful cytolytic effect (e.g. red cells are largely destroyed; myoglobin is leached out of muscle) and a solvent action on many proteins such as insulin. Whatever sulphydryl is preserved by it is probably only a fraction of that originally present. In addition, an unpredictable percentage of the sulphydryl content will be oxidized in the course of cutting, floating, and drying the sections. For this reason, a positive reaction after fixation by acid alcohol is significant, but a negative reaction is meaningless. On the other hand, mercury salts are very powerful precipitants of proteins and also form insoluble mercaptides with sulphydryl compounds. On account of these features, they are considered excellent histological fixatives, especially in combination with formaldehyde. However, Lugol's solution, used for the removal of mercury precipitates, will oxidize sulphydryls to disulphides. There is every reason to believe that sulphydryl groups produced by reduction in sections of mercury-fixed tissues will closely represent the total of sulphydryls plus disulphides

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originally present in the tissues, provided that reduction is reasonably quantitative, as it appears to be if the right reductant is employed (Mirsky and Anson, 1934–5).

SURVEY OF HISTOCHEMICAL METHODS FOR SULPHYDRYL GROUPS

Fixation. As mentioned, mercury bichloride formalin mixtures are excellent fixatives for —SH groups. However, theoretically, regular formalin should also be a good fixative since the reaction between cysteine and formaldehyde, yielding thiazolidine carboxylic acid, is readily reversible (Ratner and Clarke, 1937). Barrnett and Seligman (1954) actually found that entirely satisfactory results are obtained with plain formalin.

The liberation of sulphydryl groups. The sections, after protection with dilute collodion following the second alcohol, are run down to water and immersed in Gram-Lugol's solution in order to decompose mercaptides and thiazolidine carboxylic acid, respectively. Excess iodine is removed with a dilute solution of thiosulphate or bisulphite and the slides are washed under the tap.

For the reduction of disulphide groups, thioglycolic acid, alkalinized to pH 7 to 8 (Rudall, 1952; Barrnett and Seligman, 1954) or pH 11 (Hardy, 1952) is usually recommended. In the present studies it was found that thioglycolic acid can be replaced by thioglycerol ('thiovanol', Evans Chemetics, Waterloo, N.Y., U.S.A.), which offers the advantage that it requires no neutralization. Two to 3 ml of thioglycerol is dissolved in about 40 ml of distilled water and 10 ml of a 0·5 borate buffer at pH 8·5 to 9·1 (the latter is essentially a solution of borax) is added. The sections are left in this mixture at room temperature or at 37° for overnight. Subsequently, they are washed in several changes of large amounts of distilled water. Washing in tap water, as mentioned by Barrnett and Seligman (1954), is not recommended on account of the danger of partial oxidation, especially in cities with a chlorinated water-supply. The sections are then kept in dilute (about 0·5%) acetic acid until use (within a few hours at most).

Histochemical reactions proper. (*a*) The nitroprusside reaction, introduced by Buffa (1904), is based on a fleeting purple-red colour given by sulphydryl groups in an alkaline medium containing nitroprusside; its mechanism is poorly understood. Neither the original method nor its modification by Giroud and Bulliard (1932) gave a shade of sufficient intensity in thin paraffin sections.

(*b*) The ferric ferricyanide test (Chèvremont and Fréderic, 1943) is based on the formation of Prussian or Turnbull's blue or of a mixture of both. The composition of the blue precipitate is not definitely known since sulphydryl groups may reduce either or both components of the mixture (ferric salt and ferricyanide). However, it would appear that the reduction of ferricyanide is more important since a mixture of uranyl nitrate and ferricyanide will give a brown coloration (uranyl ferrocyanide) of identical localization but much less brilliance.

The reagent mixture must be prepared fresh but its composition is not critical. Any combination of about equal parts of 0·2 to 1% ferric ammonium sulphate and potassium ferricyanide solutions will do. Lillie and Burtner (1953) recommend a mixture of 10 ml. of 1% ferricyanide, 75 ml. of 1% ferric chloride, and 15 ml. of water. The addition of a small amount of dilute acetic acid is optional. Ferric ammonium citrate, originally recommended by the writer (1952), should not be used because in the presence of citrate the sensitivity of the method is markedly lowered, and in the case of an excess of citrate no reaction is obtained. Some batches of ferric ammonium citrate contain a large excess of citrate.

This test, as it is well known, is not entirely specific. It will stain some other reducing substances such as melanin (Staemmler, 1924) and enterochromaffin granules (Laskey and Greco, 1948) promptly and intensely. However, for reason of different localization, these substances are not likely to be confused with sulphydryl groups. A major disadvantage of the Chèvremont–Fréderic method is that it lacks a definite end-point. The longer the tissue is treated with the reagent mixture the more intense and diffuse the reaction will become. By comparison with sections in which the sulphydryl groups had been blocked it was found that the optimum compromise between intensity and specificity of the reaction is obtained by a treatment of between 3 and 6 minutes at room temperature. The sections are washed in repeated changes of dilute mineral acid, then under the tap, counterstained as desired, and mounted in balsam.

(*c*) The mercaptide reaction is based on the formation of mercaptides by the treatment of the sections with salts of heavy metals, specifically mercury or cobalt. The mercaptide is either coloured and as such directly visible or converted into black metal sulphide or demonstrated by its high absorption under the electron microscope (Bahr and Moberger, 1954).

The cobalt mercaptide method of Tahmisian and Brues (1950) was found not to yield sufficient contrast for practical purposes. The first staining method involving mercurial mercaptide was developed by Bennett (1948; but unfortunately, Bennett's reagent is not available on the market; its synthesis is difficult and the yield is very low. The writer has prepared several batches by an identical method and yet the results were not uniform. Some of the batches were excellent; others gave a rather diffuse staining of almost all tissue elements (especially of acidophil structures) without any specificity for sulphydryl groups, or no staining at all. As a rule, alcoholic solutions of good preparations had a somewhat duller, slightly brownish shade, in contrast to the brilliant orange shade of unsatisfactory batches. The writer has also synthesized a number of organic mercurials with a better shade than that of Bennett's reagent; histochemically, however, they all lacked specificity. This is very queer since in test-tube experiments they proved to be powerful inhibitors of sulphydryl enzymes. A new mercaptide method was described by Mauri, Vaccari, and Kaderavek (1954). This utilizes a water-soluble azo dye obtained by the coupling of diazotized *p*-acetoxy-mercurianiline with

H-acid in an alkaline medium. Results secured with this mercurial will be discussed later.

(*d*) The reduction of tetrazolium salts by —SH groups was first applied to histochemical use by Barrnett and Seligman (1952*a*) and subsequently used by Pearse (1953), Rogers (1953), Barrnett and Seligman (1954), and Formisano and Montagna (1954). *In vitro* studies of the reaction were published by Roberts (1954) and Findlay (1955).

Not all tetrazolium salts are suitable for histochemical purposes. Pearse noticed that triphenyltetrazolium is not reduced by tissues although it is reduced slowly in the test tube (Findlay). 2,3-diphenyl-5-methyltetrazolium is not reduced by —SH groups even in the test tube (Roberts).

Both Pearse and Barrnett and Seligman use relatively high pH values (12·8 and about 11, respectively) and add cyanide to the reagent mixture, in order to reduce —SS— groups. According to Findlay, cyanide also increases the efficiency of reduction by —SH groups. These highly alkaline solutions are somewhat destructive to tissue sections. Fortunately, it was found that neotetrazolium is reduced quite efficiently by —SH groups in the presence of cyanide at pH values as low as 8·5 to 8·8; in fact, even at 8·2 to 8·4, although at a considerably slower rate. Iodophenyl-nitrophenyltetrazolium (Rogers), which was not tried in the present studies, should be usable even around pH 7·4. However, —SS— groups are not readily reduced by cyanide below pH 11, and for this reason it is necessary to use thioglycolate or thioglycerol in a preliminary step.

By trial and error, the following method was found to yield uniformly satisfactory results:

Incubate sections reduced by thioglycerol for 1 to 2 hours at about 50° C in the following solution: dissolve 1 g of sodium cyanide in 10 to 15 ml of water; add a drop of phenolphthalein solution and enough M (6%) acetic acid to cause almost complete decolorization (about 15 ml will be needed). Add about 20 ml of a 0·5 M borate buffer at pH 8·5 to 8·8. Dissolve about 25 mg of neotetrazolium chloride in 5 ml of alcohol and pour it into the buffered cyanide solution. The mixture can be used many times. It may be kept in the refrigerator for several weeks and should be filtered before every use.

Sites of —SH groups will show up in shades of dull reddish-purple. The sections can be counterstained with haematoxylin and should be mounted in glycerol jelly.

At this point it should be mentioned that tellurous salts are also reduced by —SH compounds, with the formation of a black precipitate of elementary tellurium (Bersin and Logemann, 1933). However, this method did not work in histochemical experiments.

(*e*) Barrnett and Seligman's DDD method (1952 *a* and *b*) is based on the attachment of naphthol groups to protein sulphydryls. The naphthol is then demonstrated by azo-coupling in a second step.

It was found that the alcohol concentration of 30%, as specified by the authors, is important. Significantly higher concentrations of alcohol will result in more or less marked reduction in the intensity of the stain. The reagent mixture should be prepared fresh because stale solutions have a tendency to stain in a darker, redder shade but more diffusely and with less selectivity.

The diazonium salt recommended in the original procedure (diazo blue B, tetrazotized *o*-dianisidine) gives a variety of shades, ranging from blue to red. At sites of very high —SH concentration (hair cortex) the shade is blue; at other sites, however, it is hard to evaluate the staining in a semi-quantitative way because the equivalence of the shades is not clear. For instance, it is impossible to tell whether a dark red shade or a pale blue one is indicative of a higher —SH concentration. Actually, the variety of shades, while pleasing aesthetically, is confusing rather than informative. For this reason, it would appear that a diazonium salt giving a single shade might be preferable. A number of commercial diazonium salts proved to be usable; diazo red RC (diazotized *p*-chloro-*o*-anisidine), which yields a brilliant red hue, was generally utilized in the present work.

(*f*) Finally, a number of procedures should be mentioned which have not been tested in the present study.

Some of the methods are, no doubt, highly specific, such as the naphthol-maleimide method of Seligman, Tsou, and Barrnett (1954) or the naphthol-iodoacetamide technique of Barrnett, Tsou, and Seligman (1955). Others, like the methods adumbrated by Danielli (1950), appear potentially useful; however, they have not been put in practice so far. Techniques depending on oxidation with peracetic acid (Pearse, 1951; Lillie, Bangle and Fisher, 1953 and 1954; Scott, 1953; Braun-Falco and Rathjens, 1954) are neither sufficiently specific nor sensitive enough for the histochemical detection of —SH groups.

The problem of specificity. It is relatively easy to test histochemical methods for their specificity as —SH reagents since there is a number of good blocking reactions known.

Blockade by mercaptide formation is the least reliable of the tests. $HgCl_2$, a powerful protein precipitant, will react with —SH as well as with $—NH_2$ groups (Bjerrum, 1950). For instance, acidophilia which, no doubt, is due to $—NH_2$ groups, is distinctly reduced by treatment of the tissue with a dilute solution of a mercuric salt. Mercury blockade of both —SH and $—NH_2$ groups is reversible by —SH substances. In the present studies it was found that acidophilia suppressed by $HgCl_2$ treatment can be restored in a few minutes by amine buffers (e.g. glycine) around pH 8·5, while the —SH groups remain non-reactive after this treatment.

Conversion of —SH groups into —SS— by mild oxidants such as Lugol's solution is reasonably effective and selective (Anson, 1940). However, this method can be used only if the reagent proper does not possess a reducing action. By reduction (e.g. alkaline cyanide) the blockade is readily reversed.

Iodoacetate (Dickens, 1933; Rapkine, 1933) and ethyl-N-maleimide

(Friedmann, Marrian, and Simon-Reuss, 1949) are efficient and almost completely specific blocking agents for —SH groups, both in the test tube and histochemically. To these, chloropicrin (Rây, Guha, and Das, 1919; Chèvremont and Fréderic, 1943) (about 0·5% in 30% alcohol; 3 to 4 hours at room temperature) may be added.

It must be mentioned that alkaline cyanide solution may partially reverse the blockade effected by the last-mentioned substances, especially on greatly prolonged exposure. At 1 hour, blocked sections show no reaction at all with the tetrazolium method; from 90 minutes on there is a slowly increasing coloration of highly reactive structures (especially hair cortex), and this may attain considerable intensity in about 4 hours. However, this is not likely to cause error since a staining time of 60 to 90 minutes need never be exceeded.

Material and Methods

A large number of tissues, mainly of two species, man and the rat, was used. In the beginning, the fixative was a mercury bichloride–formalin mixture (1 part of concentrated formalin, 2 parts of a saturated solution of mercury bichloride, and 3 parts of water); however, practically all of the later material was fixed in plain 1 : 10 to 1 : 5 formalin. A number (3–12) of different tissues were processed together and embedded in a single paraffin block so that uniform treatment of all tissues was assured. The sections were handled according to the original specifications of the respective authors except for modifications as described in the preceding passages.

Results (see figs. 1 and 2)

As far as localization proper is concerned, identical pictures were obtained by all the procedures, except the method of Mauri, Vaccari, and Kaderavek, which will be discussed separately. The specificity of all methods, except the last-mentioned one, as judged by completely negative reactions after blockade, was found to be satisfactory. However, a marked difference between relative intensities of staining of various structures is observed with the individual methods. Generally speaking, Bennett's, Chèvremont–Fréderic's, and the tetrazolium method give a high contrast, some structures being intensely coloured, others much less so or not at all. With the DDD method, staining is more diffuse, and contrasts distinctly less marked. For instance, the intensely reactive beta cells of the islets of Langerhans stand out in high contrast against the unstained alpha cells with the first three methods, while with the

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Fig. 1 (plate). Pancreatic islet of guinea-pig (consecutive serial sections; not necessarily corresponding areas). Nuclear staining, wherever present in this fig. and in fig. 2, is due to haematoxylin or nuclear fast red.

A, neotetrazolium method. Beta cells stand out in good contrast. (Green filter.)
B, ferri-ferricyanide method. Good contrast. (Orange filter.)
C, Bennett's method. Good contrast. (Dark blue-green filter.)
D, DDD method. Low contrast between alpha and beta cells. (Green filter.)

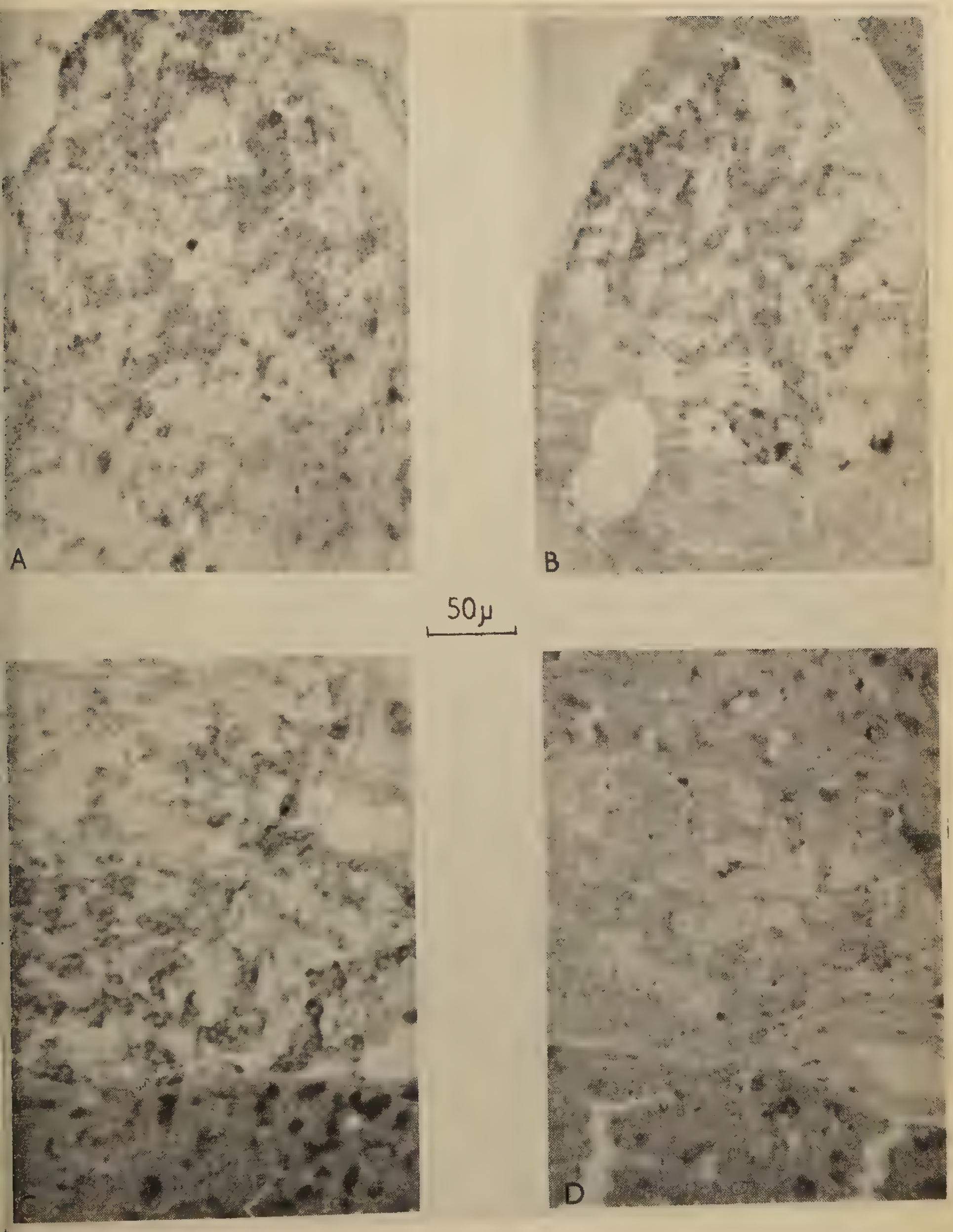

Fig. 1

G. GOMORI

DISCUSSION

According to the data presented, a number of histochemical methods can be used for the localization of protein-bound —SH groups. However, since the relative staining intensities of various structures depend on the method used, semiquantitative visual evaluation of the results is not a reliable procedure. As long as there are no independent criteria available by which it could be decided whether the high-contrast methods or the relatively low-contrast DDD technique reflect a truer picture of the actual quantitative relationships, the choice of the method to be used will be up to the personal preference of the individual worker. From a purely practical point of view, Bennett's method will be, at least for the time being, out of the reach of most laboratories, owing to the unavailability of the reagent. The ferric ferricyanide method has the disadvantage of lacking a dependable end-point. For the reasons mentioned it would appear that at present the best available histochemical methods for protein-bound —SH groups are the tetrazolium and DDD techniques.

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A Pigment in the Rat's Uterus

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With one plate (fig. 1)

SUMMARY

1. A yellowish-brown pigment was found at the old placental sites in rats killed at 10 and 20 days after littering.

2. The pigment contained ferric iron and therefore may be regarded as haemosiderin.

3. Other properties of the pigment suggest that there is also a lipid component present. Lipid is not usually associated with haemosiderin.

4. The lipid component behaves in many ways like the lipogenic pigments.

INTRODUCTION

SELYE and McKeown (1935) and Baker (1948) have noted the presence of pigment in the metrial glands of the uterus of the rat after littering. They found that it contained iron and concluded that it was haemosiderin. Baker also states that some of it stained with Sudan black in frozen sections. In a recent investigation of the *post partum* changes in the uterus of the rat (Warbrick, 1955), it was found that pigment appeared in the region of the placental sites on about the 2nd day *post partum* and that it was positive to the periodic acid / Schiff (PAS) test. As haemosiderin is not usually considered to be sudanophil or PAS-positive, this investigation was undertaken in order to study the characteristics of the uterine pigment in greater detail. Of the various techniques employed, many are such as could be expected to give a positive result with a substance containing lipid.

MATERIAL AND METHODS

Two rats were killed on the 10th day after littering and two on the 20th. On examining their uteri there was a small brown discoloration of the mesometrial part of the uterine horn at each placental site. Portions of the horns containing placental sites were removed and fixed in neutral 10% formalin for 24 hours. They were dehydrated in alcohol, cleared in cedarwood oil followed by benzene, and embedded in paraffin wax. Sections were cut, mounted, and treated with one or more of the following techniques:

1. For routine histological study, haematoxylin and eosin staining was used.
2. Staining for 10 minutes in 0·5% aqueous toluidine blue.
3. Perl's reaction for ferric iron.
4. The PAS test, with diastase-treated sections as controls.
5. The performic acid / Schiff (PFA) reaction, as described by Pearse (1951).
6. The peracetic acid / Schiff reaction, by Lillie's technique (1954).

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7. Oxidation for one hour in 4% chromic acid followed by 45 minutes in Schiff reagent.

8. Exposure of section without previous oxidation to Schiff's reagent for periods up to 18 hours.

9. Staining in a saturated solution of Sudan black in 70% alcohol for 3 hours, rinsing in 70% alcohol, and mounting in glycerine jelly.

10. The long Ziehl–Neelsen method as employed by Pearse (1953).

11. Gomori's chrome alum / haematoxylin method.

12. Mallory's technique for haemofuscins.

13. The ferric ferricyanide reduction test, by Lillie's method (1954).

14. (*a*) Bromination, and (*b*) treatment with 5% chromic acid for 1 hour, followed by the ferric ferricyanide reduction test.

15. The Masson–Fontana alkaline silver technique.

16. The Gmelin test for haematoidin.

17. Bleaching in hydrogen peroxide for 48 hours.

18. Exposure to ultra-violet light for fluorescence.

OBSERVATIONS

The distribution, appearance, and properties of the pigment were the same in all the specimens that were examined, whether from the 10-day or 20-day rats. Pigment was found in large quantities at each of the old placental sites. All the pigment was intracellular and the cells containing pigment were grouped either in the remains of the metrial gland situated in the mesometrial triangle or in the mesometrial half of the endometrium, where they lay close to the myometrium (fig. 1, A). The pigment was yellow to light brown and formed granules that varied in size from fine particles to those that were 10 or more microns in diameter (fig. 1, B).

In assessing the results of the various techniques that were employed, allowance must be made for the original colour of pigment, because it is on to this that any second colour is superimposed. For instance, a method which gives a blue colour as a positive may with the pigment produce a green or greenish-blue shade. The observations described in detail below are summarized in table 1 (see p. 15).

The pigment was not stained by either haematoxylin or eosin, but with toluidine blue it became a deep greenish-blue, in parts appearing almost black. Perl's test for ferric iron gave a strong positive reaction and resulted in all the pigment granules becoming a deep blue, while a lighter bluish-green shade was imparted to the cytoplasm of the cells containing pigment. The pigment reacted strongly with the PAS technique, the granules becoming a deep brownish-red which could be readily distinguished from their normal colour. The same colouring of the pigment was also found in the diastase controls,

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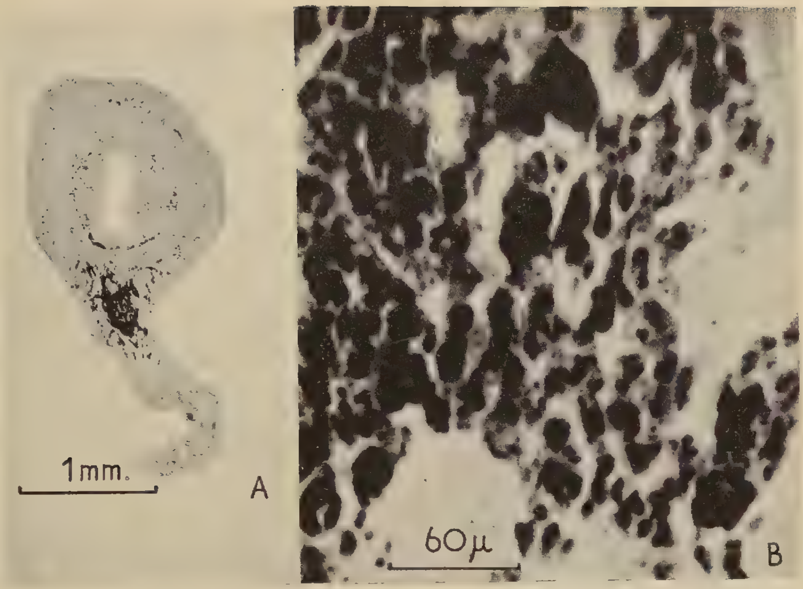
FIG. 1 (plate). A, transverse section of a uterine horn of a rat killed 20 days after littering. Note the distribution of the black pigment. B, a portion of A, at higher magnification.



J. G. WARBRICK

and thus the possible presence of glycogen was excluded. The pigment remained uncoloured after exposure to Schiff's reagent for 18 hours when no previous oxidation was employed. This shows that the PAS method was giving a true positive reaction and was not responding to the presence of preformed aldehyde groups in the pigment. The PFA reaction, the peracetic acid / Schiff reaction, and oxidation with chromic acid followed by Schiff's reagent all failed to produce any colouring of the pigment. It was sudanophil, most of the particles giving a greyish colour with Sudan black, although some of the larger ones became a deep black. It was acid-fast, resisting the acid alcohol differentiation in the long Ziehl–Neelsen method, so that while the background was a pale pink the granules were a deep brownish-red. With the chrome alum / haematoxylin technique of Gomori the pigment took up some of the stain, and although the original yellow colour was not completely abolished, the effect of the haematoxylin could be easily recognized. After alcoholic differentiation in Mallory's technique for haemofuscins some of the basic fuchsin was retained by the pigment granules, which became a light reddish-brown. With the ferric ferricyanide reduction test the section as a whole was coloured a pale green but the pigment was more markedly affected. Nearly all the granules were coloured a deep green while some of the larger ones became a very deep greenish-blue. A positive reaction with this test is normally indicated by a blue colour. Nevertheless, the granules are regarded as being positive because they colour (although for the most part in green) much more deeply than the background. After bromination and the chromic acid treatment the granules remain uncoloured by the ferric ferricyanide method. Treatment with alkaline silver resulted in slight darkening of the granules which became browner. The Gmelin test for haematoidin was negative while the pigment was not bleached by hydrogen peroxide. There was no fluorescence under the microscope when ultra-violet light was employed.

Discussion

This investigation confirms the findings of Selye and McKeown and of Baker that the pigment contains ferric iron. These writers were also undoubtedly right in regarding the pigment as haemosiderin as this substance is by definition a pigment which exhibits one or more of the reactions of ferric iron (Lillie, 1954). Also the uterine pigment resists bleaching by hydrogen peroxide and this is a characteristic of haemosiderin. According to Lillie (1954), three varieties of haemosiderin can be recognized, one of which stains with basic dyes, as does the uterine pigment with toluidine blue. Haemosiderin is one of the breakdown products of haemoglobin (Florey, 1954) and the pigment in the rat's uterus is certainly derived from the blood, as it is found almost entirely at the old placental sites into which haemorrhage occurs at the time of separation of the placenta.

Unlike the pigment in the rat's uterus, human haemosiderin is not PAS-positive (Lillie, 1950). According to Pearse (1953) haemosiderin is not acid-fast, does not stain with Sudan black nor colour with Gomori's chrome alum / haematoxylin technique, is negative in the ferric ferricyanide test, and does not darken alkaline silver. All these techniques as well as Mallory's technique for haemofuscins give positive results when applied to the uterine pigment. It is thus clear that while the uterine pigment may be regarded as a haemosiderin, it possesses several properties that are not usually associated with this substance.

The colouring with Sudan black suggests that there is a lipid element present, and other positive reactions, as with the PAS reaction and the Ziehl–Neelsen technique support this. A comparison may be made between the uterine pigment and the lipogenic pigments, the properties of which have been summarized by Gomori (1952), Pearse (1953), and Lillie (1954). These pigments, which are fairly widely distributed, are known by a variety of names, such as ceroid, luteolipin, wear and tear pigment, and lipofuscin. They all arise as oxidation products of a lipid precursor (Pearse, 1953). Their reactions with different histological and histochemical techniques vary and this may be related to the degree of oxidation that they have undergone. Like the pigment in the rat's uterus they withstand lipid solvents and can be identified in paraffin sections. Again, many of the lipogenic pigments such as adrenal lipofuscin are acid-fast and PAS-positive, react with ferric ferricyanide, and colour with oil-soluble colouring agents in paraffin sections. They may darken with alkaline silver, colour with Gomori's chrome alum / haematoxylin, and retain basic fuchsin in Mallory's technique. In these respects the uterine pigment resembles them. It differs from some such as ceroid which is positive to peracetic acid / Schiff and fluoresces in ultra-violet light. Nor are the lipogenic pigments usually associated with iron, although ceroid may occasionally contain a few granules of pigments containing iron.

The negative Gmelin reaction indicates that haematoidin, the iron-free breakdown product of haemoglobin, is absent. Although the uterine pigment in some respects resembles melanin, it is not melanin, for granules of the latter substance are darker in colour, do not stain with Sudan black nor give the PAS-reaction, and are usually bleached within 48 hours by hydrogen peroxide.

It thus appears that the uterine pigment should be regarded as a variety of haemosiderin in which the substance containing iron is mixed or perhaps combined with a lipid component that has many properties in common with the lipogenic pigments.

I wish to thank Professor G. M. Wyburn for his helpful advice. Part of the cost of the investigation was defrayed by a grant from the Misses Cruden Fund.

TABLE 1

Technique employed	*Colour of pigment*	*Comments*
Haematoxylin and eosin	unchanged	. .
Toluidine blue	deep greenish-blue	basiphil with a basic dye
Perl's method	deep blue	ferric iron present
Periodic acid / Schiff	brownish-red	a strong positive
Schiff's reagent	unchanged	no preformed aldehyde group present
Performic acid / Schiff	unchanged	negative
Peracetic acid / Schiff	unchanged	negative
Chromic acid / Schiff	unchanged	negative
Sudan black	grey to deep black	suggests presence of a lipid
Long Ziehl–Neelsen	brownish-red	acid fast suggesting a lipid
Gomori's chrome alum / haematoxylin	greenish-blue	a positive reaction
Mallory's technique	pale reddish-brown	a weak positive
Ferric ferricyanide	green to greenish-blue	a positive reaction
Bromination and ferric ferricyanide	unchanged	negative
Chromic acid and ferric ferricyanide	unchanged	negative
Alkaline silver	brown	a weak positive
Gmelin test	unchanged	no haematoidin present
Hydrogen peroxide	unchanged	an absence of bleaching
Fluorescence (U.V.)	. .	no fluorescence observed

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The Site of Damage in Amoebae Exposed to Lethal Concentrations of Methyl di-(β-Chloroethyl)-Amine (a 'Nitrogen Mustard')

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SUMMARY

The technique of nuclear transfer in *Amoeba proteus* was used to study the extent to which methyl di-(*β*-chloroethyl)-amine damages nucleus and cytoplasm. Results obtained showed that cytoplasm was damaged directly by the drug; exposure of normal cytoplasm to nuclei damaged by *β*-chloroethylamine for up to 3 days caused no lethal damage. Nuclei were probably directly damaged, but could also be damaged by injured cytoplasm, normal nuclei being lethally damaged when exposed to injured cytoplasm for only 2 hours. Death from lethal cytoplasmic damage generally occurred within a few days; animals with lethally damaged cytoplasm seldom divided. Death from lethal nuclear damage was delayed in some cases for as long as 6 weeks, such nuclei being able to divide once or twice before death.

INTRODUCTION

WHEN tissues are treated with a 'nitrogen mustard', nuclear disturbances such as chromosome breaks and spindle failure are easily observed (e.g. Koller, 1947; Dustin, 1947; Auerbach and Moser, 1950; Loveless and Revell, 1949).

It has also been suspected that the nitrogen mustards damage the cytoplasm, but it has been difficult to assess the extent of cytoplasmic damage. Although it has been thought likely that the nuclear damage was a direct action of the drug, the possibility remained that the nuclear damage might be secondary and caused by damage to the cytoplasm. We have used the technique of nuclear transfer in *Amoeba proteus* (Lorch and Danielli, 1950, 1953) to study the extent to which the nitrogen mustard, methyl di-(*β*-chloroethyl)-amine (MBA), damages nucleus and cytoplasm.

EXPERIMENTAL

The culture methods were similar to those used by Lorch and Danielli (1953). Amoebae which had been experimented upon were kept singly in small solid watch-glasses containing 1–2 ml of Chalkley's medium, together with *Colpidium*. Every 3 or 4 days the amoebae were transferred to clean dishes with a fresh supply of medium and *Colpidium*. Control amoebae grew well and divided for five or six generations under these conditions. Most of the clones formed were discarded when about thirty animals were present, but about 10% of these small clones were grown in large dishes to mass cultures of several thousand animals.

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Nuclear transfer was carried out by a technique similar to that described by Lorch and Danielli (1953). At least 80% of the transfers made on otherwise normal animals were successful in the sense that the animal divided at least once, and of those animals which divided once at least 95% went on to give mass cultures.

The nitrogen mustard was supplied by Boots Pure Drug Company in small sealed bottles each containing 5 mg in solid form. The bottles were kept in the dark. Immediately before use, 5 ml of 0·02% aqueous sodium citrate were added. The solution was used within 2–3 minutes after preparation, for there was a significant fall in toxicity after standing for 30 minutes. Dilutions were carried out with 0·02% sodium citrate solution.

Amoebae were treated with the drug in groups of 20. Each group was exposed to 1 ml of a known concentration of drug for 15 minutes, unless otherwise stated. They were transferred with a fine pipette from the drug solution to Chalkley's solution and left for 5 minutes. This washing was repeated in a second lot of Chalkley's solution and then the animals were placed in Chalkley's solution in a clean watch-glass, with food. Some were used for transfers and others were placed singly in watch-glasses and observed periodically.

The buffering solution, 0·02% sodium citrate, had a pH of 7·3±0·1. After the addition of the highest concentration of nitrogen mustard used in the present experiments the pH fell to 7·0. The pH values of cultures lay in the range 7·0±0·3. pH values were determined with a glass electrode. Control experiments showed that exposure to sodium citrate solution had no significant effect on subsequent success or on the after effects of nuclear transfer.

NOMENCLATURE

We have followed the scheme suggested by Lorch and Danielli (1950). Thus N_nN_c represents a normal animal, i.e. unoperated and with normal cytoplasm and nucleus. $N_n \rightarrow N_c$ represents a transfer of a normal nucleus to a normal cytoplasm, and the resultant animal is represented as $\overrightarrow{N_nN_c}$. Nuclei and cytoplasms treated with the nitrogen mustard (MBA) are indicated by MBA_n and MBA_c respectively.

EXPERIMENTAL RESULTS

The effect of variation in temperature

The results of treating amoebae with a nitrogen mustard are influenced both by the temperature at which the animals are cultured and by the temperature of the drug solution at the time of exposure. Amoebae cultured at temperatures above 20° C are difficult to operate upon because of a great increase in elasticity of the cortex. Amoebae exposed to a nitrogen mustard solution at a temperature of 24° C or above tend to disintegrate during the experiment. Fig. 1 shows the effect of exposure of amoebae from the same culture to 0·005% nitrogen mustard for 15 minutes at 4°, 17°, 24°, and 28° C. After washing, the animals were kept at about 23° C. At the lowest temperature about 80% of the animals survived and were able to form mass cultures. At the highest temperature all the animals were dead in 24 hours.

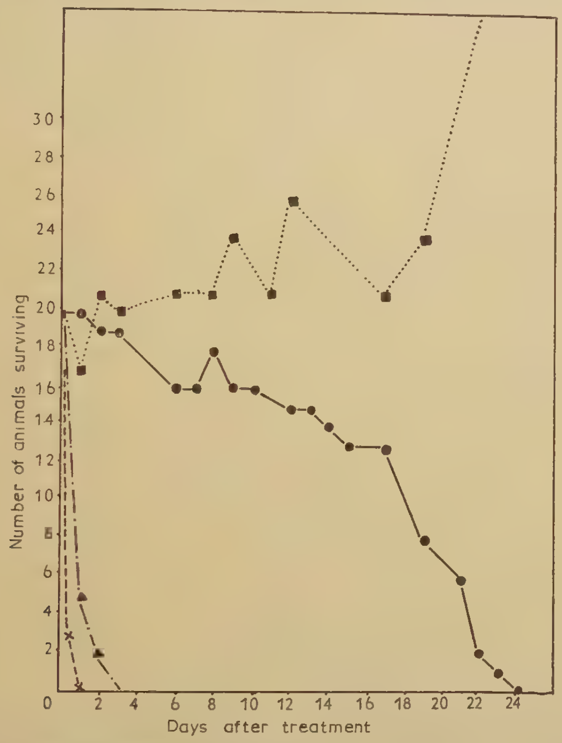

FIG. 1. Survival curves for *Amoeba proteus* exposed to 0·005% nitrogen mustard at 4° C (■), 17° C (●), 24° C (▲), and 28° C (×).

The effect of variation in nutrition

The toxicity of the mustard was also strikingly influenced by the state of nutrition of the amoebae. Fig. 2 shows a survival curve for animals of the same clone in different nutritional states. All were exposed to 0·005% mustard. One group was taken directly from a mass culture: all were dead 17 days after exposure to MBA. A second group was kept in a watch-glass with very scanty food for 3 days before exposure: all were dead 5 days after exposure. A third group was kept in a watch-glass, also for 3 days, but with abundant food: very few had died by the 17th day after exposure and the last did not die until the 50th day.

The effect of variation in nitrogen mustard concentration

Unfortunately the means at our disposal did not enable us to standardize both temperature and nutrition to a sufficient degree to preclude variation in results of exposure to the drug caused by these factors. But it was found that this disadvantage could be nullified for most purposes by grouping the animals according to the time taken for the drug to kill all the animals exposed to a given mustard concentration in an individual experiment. If all the animals were killed in 4 days, all were included in group I; if in 5–15 days in group II, and if any survived for more than 15 days all were included in group III.

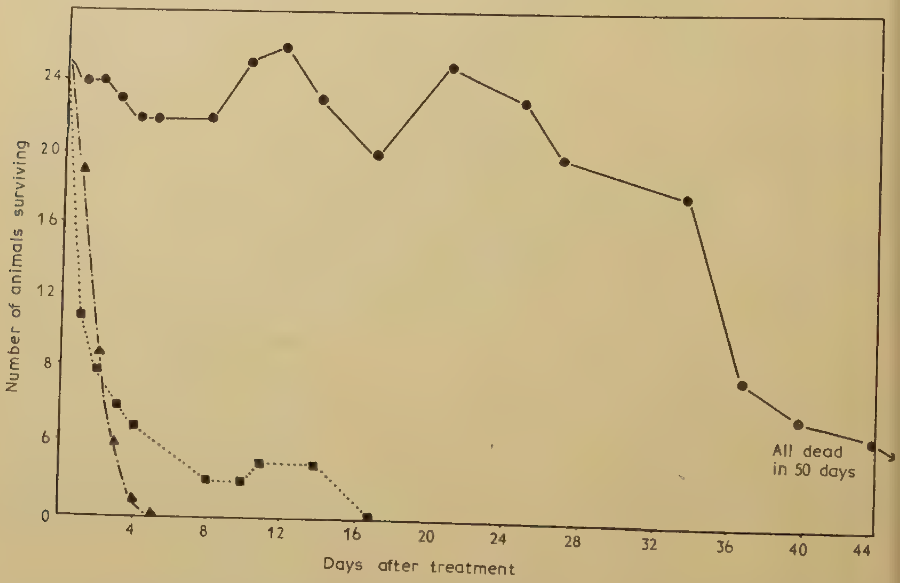

Fig. 2. Survival curves for amoebae of the same clone in different nutritional states exposed to 0·005% nitrogen mustard; amoebae directly from large culture (■), amoebae kept in small dishes with abundant (●) and scanty (▲) food.

A normal amoeba, when transferred to a clean watch-glass, quickly attaches itself to the glass-water interface and is commonly in motion. Amoebae in group I never became attached and soon became clavate or spherical and cytolysed. In group II a small proportion of the animals became attached after an interval of 3–4 days, and about 2% were able to divide. In 25–50% the nuclei became swollen, in some instances a few hours after exposure, but in others not for 1–2 days. All ultimately became clavate or spherical, and cytolysed. Animals in group III also remained floating for several days, after which about 60% became attached and divided. After 2–3 weeks all became detached and sluggish, and eventually either became spherical and cytolysed or shrank and disintegrated. Fig. 3 shows survival curves for experiments falling into all three groups.

Many of the animals in group III were able to divide at least once, but there was a substantial delay in division (fig. 6, see p. 27).

The effect of a normal nucleus upon treated cytoplasm

The results described in the previous section show that 0·002% MBA killed 100% of the amoebae. The transfer $N_n \rightarrow MBA_c$ was used to investigate the extent to which the cytoplasm may recover when given a normal nucleus. Table 1 sets out the results of about 150 transfers carried out on animals after exposure to MBA concentrations in the range 0·02–0·002. Of these animals 15% divided at least once, 14% at least twice, and 13% went on to give mass cultures. Thus about 90% of animals able to divide once give mass cultures. When normal amoebae are set out singly in watch-glasses, about 5–10 % fail to establish mass cultures. Consequently it may be concluded that if a cytoplasm recovers sufficiently to be able to divide, then it is almost certain to make a complete recovery, provided the nucleus is normal.

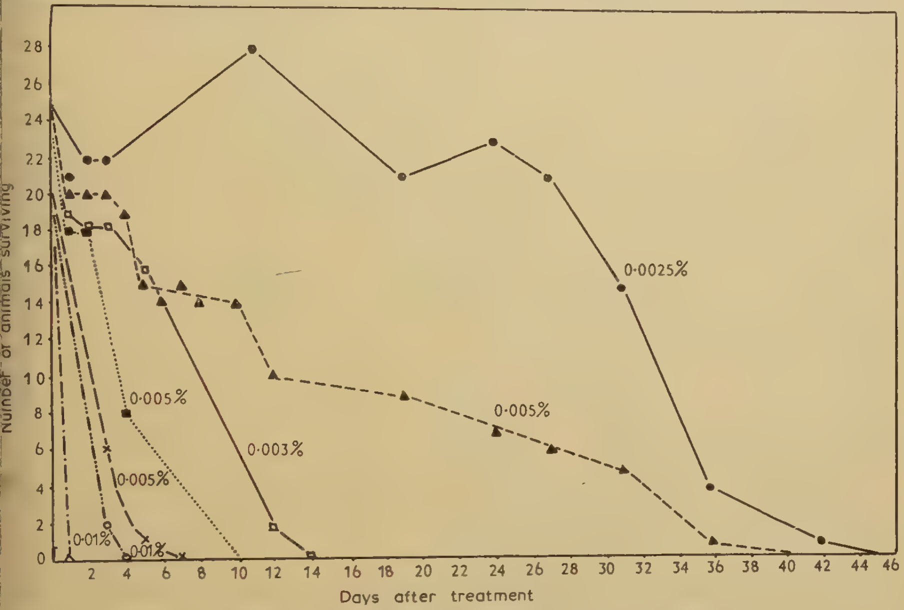

FIG. 3. Survival curves after exposure to 0·01–0·0025% nitrogen mustard showing the variation in response obtained using different cultures of amoebae.

In table 1 the results are divided into groups I, II, and III. Only animals in group III, i.e. animals, the control set for which lived more than 15 days, showed a high proportion of complete recovery, but in group III the effect of nuclear transfer is striking: whereas only 3% of unoperated animals (MBA_n MBA_c) gave mass cultures, 33% of $\overrightarrow{N_n MBA_c}$ recovered completely.

The effect of normal cytoplasm upon treated nuclei

Nuclei of animals which had been treated with MBA were transferred to cytoplasms obtained by enucleating normal amoebae, giving $\overrightarrow{MBA_n N_c}$. The results are represented in table 1. Of the animals which fell into group I, 6% divided once, and none twice. In group III, 51% divided once and 16%

divided twice, but only 5% gave mass cultures. When these results are compared with those obtained for $\overrightarrow{N_n\mathrm{MBA}_c}$ it is evident that after a given exposure to MBA, damage to a nucleus interferes less with a subsequent division than does damage to a cytoplasm. But a degree of nuclear damage which permits division may still be lethal, whereas if a damaged cytoplasm once divides it will normally recover completely. In the long run transfer of damaged nuclei to normal cytoplasm does not increase their survival, whereas transfer of normal nuclei to damaged cytoplasms may produce a large increase in survival rate.

TABLE I

The percentage of amoebae which divided, and which formed mass cultures, after treatment with MBA, *and the effect of nuclear transfer upon these processes. The data in the table are for amoebae treated with* MBA *concentrations lying between 0·02 and 0·002%*

	Group	*% dividing once*	*% dividing twice*	*% forming mass cultures*
Unoperated amoebae	I	0	0	0
	II	0	0	0
	III	50	18	3
$\mathrm{MBA}_n \rightarrow N_c$	I	6	0	0
	II	15	3	3
	III	51	16	5
$N_n \rightarrow \mathrm{MBA}_c$	I	4	4	4
	II	6	4	3
	III	36	35	33

The cause of death in fatally injured amoebae

(i) *Death caused by cytoplasmic damage.* From the results already presented it is clear that both nuclei and cytoplasms may be fatally injured, separately. The question arises: In any given case is death primarily due to nuclear or to cytoplasmic damage? To elucidate this point, data are presented in fig. 4 for amoebae which died, either without dividing (curves 1, 2, and 3) or after dividing (curves 4 and 5).

Comparison of unoperated amoebae (curve 1) with operated animals (curves 2 and 3) shows that the transfer $N_n \rightarrow \mathrm{MBA}_c$ diminishes duration of life of lethally damaged cytoplasm, but that $\mathrm{MBA}_n \rightarrow N_c$ increases the expectation of life of the nucleus. Further, whereas a significant number of unoperated (curve 4) and $\mathrm{MBA}_n \rightarrow N_c$ (curve 5) divided at least once before dying, none of the group $N_n \rightarrow \mathrm{MBA}_c$ did so. The following deductions may be made:

(*a*) Lethal cytoplasmic damage causes death within 5 days of damage in most cases.

(*b*) If cytoplasm is lethally damaged it cannot divide, even with a new nucleus: if cytoplasm is able to divide once, it does not die unless the nucleus is lethally damaged.

(*c*) Lethal nuclear damage usually causes death much more slowly than does cytoplasmic damage.

(*d*) If death occurs within four days of exposure to MBA, it is caused mainly by cytoplasmic damage, whereas death after five days is caused mainly by nuclear damage.

These deductions are supported by the results obtained by study of amoebae which were enucleated before treatment (fig. 5). Curve 1 is for untreated enucleated cytoplasms, of which 50% died in 9 days. Curves 2 and 4 are for cytoplasms after exposure to 0·01 and 0·005% MBA respectively. Curves 3 and 5 are for normal amoebae treated in the same manner. In this experiment 0·01% MBA killed about 90% of the animals in 1 day and 100% in 2 days, and 0·005% MBA killed about 50% in 1 day and 100% in about 8 days: there was no significant difference between nucleated and enucleated animals. In a further experiment both enucleated cytoplasms and nucleated amoebae were exposed to MBA: the cytoplasms were renucleated, and the nucleated animals were enucleated and then renucleated. In both cases the renucleation was with normal nuclei. The renucleation made no significant difference.

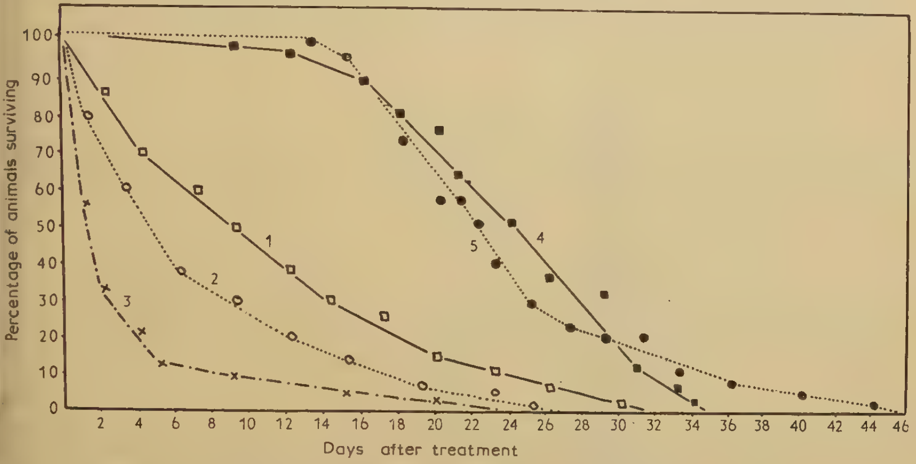

FIG. 4. Death-rate curves for amoebae treated with nitrogen mustard. Curves 1, 2, 3 are for amoebae which were unable to divide after treatment: 1, $\overrightarrow{MBA_nN_c}$; 2, MBA_nMBA_c; 3, $\overrightarrow{N_nMBA_c}$. Curves 4 and 5 are animals which divided once or twice but failed to form mass cultures: 4, $\overrightarrow{MBA_nN_c}$; 5, MBA_nMBA_c.

These experiments prove that the presence of a nucleus, either during exposure or after exposure, makes no difference to the life of an amoeba treated with 0·01% MBA, so that death must be caused by the cytoplasmic damage, and this cytoplasmic damage is caused directly by the MBA and is not a consequence of nuclear damage.

(ii) *Death caused by nuclear damage.* The fact that, during and after exposure to certain concentrations of MBA, the presence of a nucleus makes no difference to the duration of life, shows beyond doubt that these concentrations cause lethal damage to the cytoplasm. But whereas cytoplasms will survive for many days when enucleated, nuclei will not remain viable when isolated. Hence we could not expose isolated nuclei to MBA and thus discover whether nuclei are damaged directly or as a result of cytoplasmic injury. All that could be done was to transfer nuclei as soon as possible after exposure of whole amoebae to MBA.

To reduce the interval between exposure and transfer, an exposure of 2 minutes to 0·13% MBA was used. Preliminary experiments showed that this produced damage equal to that sustained in 15 minutes with 0·01% MBA. This exposure was followed by two rinses, each of 1 minute, in Chalkley's solution, followed by transfer of the nucleus to a normal cytoplasm. Table 2 shows the results obtained by such experiments, in which the interval from beginning of exposure to MBA to completion of transfer varied from 8 minutes to 3 hours. The control unoperated amoebae (MBA_nMBA_c) all died within 5 days, probably from cytoplasmic damage. Nuclei transferred within 30 minutes of exposure lived for an average of about 26 days, whereas if transferred after 3 hours they lived for an average of only 17 days. From these results it appears that the damage to nuclei occurs in two stages, one of which is complete within 10 minutes of exposure and is probably a direct action of the nitrogen mustard, while the other is becoming apparent after 3 hours' exposure to lethally damaged cytoplasm, and is presumably a result of the cytoplasmic damage.

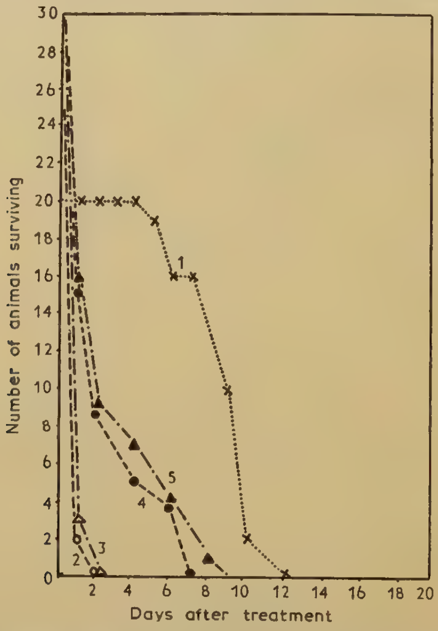

FIG. 5. Survival curves obtained when nucleate (—.—) and enucleate (– – – – –) amoebae were treated with 0·1% (△ & ○) and 0·005% (▲ & ●) nitrogen mustard. The last curve (× . . . . . . ×) is a survival curve for untreated enucleate amoebae.

Indirect damage to nuclei and to cytoplasm

The experiments described above indicate that, when exposed to MBA under conditions just sufficient to cause lethal damage, the MBA damages nucleus and cytoplasm directly, separately. But the results also indicate that damaged nuclei may injure normal cytoplasm, and conversely. The following experiments were designed to estimate how quickly this indirect action becomes lethal. In these experiments the principle used was to expose either normal nuclei or normal cytoplasm to cytoplasm or nuclei respectively which had been lethally damaged by MBA. This exposure was for a given time, after which the normal component which had been exposed to the component damaged by MBA was either restored to normal cytoplasm or given a normal nucleus.

TABLE 2

The effect of transferring nuclei from amoebae exposed to 0·13% MBA *for 2 minutes to normal cytoplasms at different intervals after beginning the exposure to* MBA

Interval before transfer	*Number of animals*	*Number dividing*	*Average life in days with standard deviation*	*% survival*
8–10 minutes	8	2	28 ± 10	0
11–15 ,,	6	1	21 ± 8	0
16–30 ,,	8	3	21 ± 10	0
3 hours	10	1	13 ± 9	0
Unoperated animals	10	1	5 ± 1	0

(i) *The action of damaged nuclei upon normal cytoplasm.* Amoebae were exposed to 0·01% MBA. If not operated upon, all these animals would have died in 3 days. Nuclei from such animals were transferred to normal cytoplasms to form $\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c}$: the resulting animals live for up to 35 days or more, but all die eventually. After either 1 or 3 days the MBA_n was removed and a normal nucleus reinserted to give $\overrightarrow{\mathrm{N}_n\mathrm{N}_c}$. The series of operations was thus

$$\mathrm{N}_n\mathrm{N}_c \rightarrow \mathrm{N}_n \text{ (discarded)} + \mathrm{N}_c$$

$$\mathrm{N}_c + \mathrm{MBA}_n\mathrm{MBA}_c \rightarrow \overrightarrow{\mathrm{MBA}_n\mathrm{N}_c} + \mathrm{MBA}_n \text{ (discarded)}$$

$$\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c} \rightarrow \mathrm{MBA}_n \text{ (discarded)} + \mathrm{N}_c^*$$

$$\mathrm{N}_c^* + \mathrm{N}_n\mathrm{N}_c \rightarrow \overrightarrow{\mathrm{N}_n\mathrm{N}_c^*} + \mathrm{N}_c \text{ (discarded)}$$

This series involved two enucleations and two renucleations. When this series of operations is performed without MBA, i.e. with normal amoebae, 60% of the animals form mass cultures: that is the double nuclear transfer causes a loss of 40% of the animals. In the experiment with MBA, 60% of the animals $\overrightarrow{\mathrm{N}_n\mathrm{N}_c^*}$ formed mass cultures. It follows that exposure of normal cytoplasm to a lethally damaged nucleus for up to three days causes no lethal damage to the cytoplasm.

(ii) *The action of damaged cytoplasm upon normal nuclei.* When a normal nucleus is inserted into a cytoplasm which has been exposed to 0·01% MBA, to give $\overrightarrow{\mathrm{N}_n\mathrm{MBA}_c}$, it usually swells and after about 2 hours becomes spherical and relatively clear. These swollen nuclei collapse when pricked with a needle, whereas undamaged nuclei show no visible changes when pricked with a fine needle. It was difficult to return these nuclei to normal cytoplasm, and in the few instances in which this was accomplished (to give $\overrightarrow{\mathrm{N}_n^*\mathrm{N}_c}$), the amoeba failed to divide. Thus 2 hours' exposure to cytoplasm damaged with this high concentration of MBA was lethal to the nucleus.

In a further experiment amoebae were exposed to a lower concentration of MBA, 0·004% (table 3). If not operated upon, these animals died after an average life of 20 days. When renucleated to give $\overrightarrow{\mathrm{N}_n\mathrm{MBA}_c}$, 25% lived, showing that 75% of the cytoplasms were lethally damaged or were sufficiently abnormal to damage the nuclei lethally. If the nuclei were restored to normal cytoplasm after 2 days in MBA_c, 26% of the animals so formed ($\overrightarrow{\mathrm{N}_n^1\mathrm{N}_c}$) survived. This showed that 2 days' exposure to a lethally damaged cytoplasm also lethally damaged the nucleus.

TABLE 3

The effect upon normal nuclei (N_n) *of cytoplasm which had been damaged by* 0·004% MBA. N_n^1 *indicates nuclei which had been present in* MBA_c *for two days before retransplantation to* N_c

Type	*Number*	*% first divisions, corrected for operation injury*	*Average life of animals killed* (days)	*% survival corrected for operation injury*
$\mathrm{MBA}_n\mathrm{MBA}_c$	10	40	20	0
$\overrightarrow{\mathrm{N}_n\mathrm{MBA}_c}$	10	25	10	25
$\overrightarrow{\mathrm{N}_n^1\mathrm{N}_c}$	12	26	19	26

The action of MBA *upon division*

After exposure to MBA sufficient to kill amoebae, the animals often divide once, but seldom do so twice before dying. Discussion here will therefore be limited to the first division. Fig. 6 shows curves for division of normal amoebae ($\mathrm{N}_n\mathrm{N}_c$), normal amoebae after nuclear transfer $\overrightarrow{\mathrm{N}_n\mathrm{N}_c}$, animals treated with MBA ($\mathrm{MBA}_n\mathrm{MBA}_c$), normal nuclei in treated cytoplasm ($\overrightarrow{\mathrm{N}_n\mathrm{MBA}_c}$), and treated nuclei in normal cytoplasm ($\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c}$).

Comparison of $\mathrm{N}_n\mathrm{N}_c$ with $\overrightarrow{\mathrm{N}_n\mathrm{N}_c}$ shows that the operation of transfer causes an average delay of about 5 days. Simple treatment with MBA causes a delay of similar magnitude. When transfer and exposure to MBA are combined, there is a total delay of about 8 days, by comparison with N_nN_c. This delay of 8 days is found both with treated nuclei and treated cytoplasm.

A detailed examination of division rates will be published elsewhere, and an account of abnormalities in division will be included in a paper discussing the action of lower concentrations of MBA (Ord, 1955).

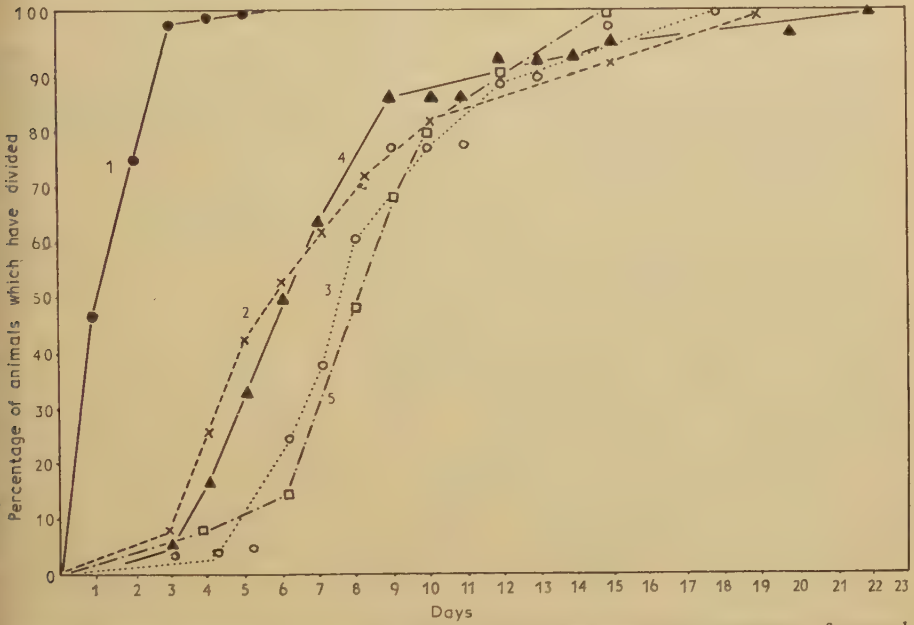

FIG. 6. Curves showing the time of first division after separation or treatment of normal amoebae and those treated with nitrogen mustard. 1, control amoebae N_nN_c (—●—); 2, control transfers $\overrightarrow{N_nN_c}$ (- - -×- - -); 3, MBA_nMBA_c (- - ○ - -); 4, $\overrightarrow{MBA_nN_c}$ (—▲—); 5, $\overrightarrow{N_nMBA_c}$ (–.–□–.–).

DISCUSSION

The results obtained in this work show that amoeba cytoplasm is damaged lethally by an appropriate exposure to MBA, and that this damage is not a result of nuclear damage. A normal cytoplasm may be exposed for at least 3 days to a lethally damaged nucleus without itself suffering lethal damage. If the cytoplasm is lethally damaged, it usually dies within 4 days. If cytoplasm is able to divide once it usually recovers completely.

On the other hand, nuclei also probably suffer damage which is a direct effect of MBA, and not a result of cytoplasmic damage. Nuclei, however, are much more readily damaged by injured cytoplasm than cytoplasm is by an injured nucleus. Thus even 2 hours' exposure to a lethally damaged cytoplasm may lethally damage a nucleus. Nuclear damage by a given concentration of MBA takes longer to kill the amoeba than does cytoplasmic damage—perhaps five or tenfold longer. Also, whereas an animal with lethally damaged cytoplasm (i.e. $\overrightarrow{\mathrm{N}_n\mathrm{MBA}_c}$) usually does not divide, if it has a normal cytoplasm and a damaged nucleus (i.e. $\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c}$) it is likely to divide several times before dying.

Thus nuclear and cytoplasmic injuries by MBA are quite distinct and independent phenomena.

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The Site of Damage in Amoebae Exposed to X-Rays

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SUMMARY

The technique of nuclear transfer was used to study the extent to which X-rays damage nuclei and cytoplasms of *Amoeba proteus*. Results showed that cytoplasm was directly damaged by the radiations. Cytoplasmic damage with doses below 280,000 r was reversible, though damaged cytoplasm could lethally damage normal nuclei. Damage with doses above 300,000 r caused lethal damage and death occurred without division in 0–3 days. Damage to nuclei was probably a combination of a direct action of the radiations and exposure to reversibly damaged cytoplasm. Nuclei were 2·4 times as sensitive as cytoplasms, the LD_{50} doses being approximately 120,000 r for nuclei and 290,000 r for cytoplasm. Death due to nuclear damage generally occurred without division within 3 to 6 weeks. Nuclear damage cannot be interpreted in terms of the 'target' theory.

EXPOSURE of cells to radiations is known to produce both nuclear and cytoplasmic changes. It has been difficult to assess the separate extent of either nuclear or cytoplasmic damage. In this work, using the technique of transferring nuclei from one amoeba to another (Commandon and de Fonbrune, 1939; Lorch and Danielli, 1950), we have attempted to study both the direct and indirect action of X-rays on nuclei and on cytoplasms. We have used methods similar to those described in our work on the effect of a 'nitrogen mustard' on nuclei and cytoplasms (Ord and Danielli, 1956; Ord, 1956).

METHODS

All experiments were done with *Amoeba proteus*. In all but two experiments (see text) X-rays were given with a 400 kVp, 5 mA machine. The amoebae were irradiated in Chalkley's solution in small glass vials 7·5 or 15 mm in diameter, at a distance of 9 or 9·5 cm from the source and at an intensity of 3,300 or 3,000 r/minute. The time of exposure was varied to give doses ranging from 5,000 to 350,000 r.

Transfers of irradiated nuclei to control enucleated cytoplasms and of control nuclei to irradiated enucleated cytoplasms were done 10 minutes to 48 hours after irradiation by means of a de Fonbrune micromanipulator. Control transfers (that is, transfers of a normal nucleus to a normal cytoplasm) were 80–90% successful in forming mass cultures after the operation. The technique of nuclear transfer and the method of culturing amoebae have been described elsewhere (Lorch and Danielli, 1950, 1953). After treatment both operated and control amoebae were grown singly in small solid watch-glasses and kept until they either died or formed clones of 20–30 individuals. Damage was estimated from (1) behaviour after irradiation, e.g. attachment, movement, and feeding; (2) changes in viscosity of nucleus and cytoplasm and in the properties of the cytoplasmic membrane as observed during microdissection;

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(3) the length of delay between irradiation and first division, and between first and second divisions; (4) the ability to form clones of 20–30 individuals; (5) length of life when damage was lethal.

Nomenclature is according to the scheme of Lorch and Danielli (1950) and Ord and Danielli (1956); that is, transfer of an irradiated nucleus to normal cytoplasm has been referred to as $X_n \rightarrow N_c$, the resultant animal being $\overrightarrow{X_nN_c}$; the transfer of a normal nucleus to irradiated cytoplasm as $N_n \rightarrow X_c$, the resultant animal being $\overrightarrow{N_nX_c}$; an unoperated irradiated amoeba would then be X_nX_c.

EXPERIMENTAL RESULTS

I. *The general effects of X-rays*

In this work all experiments were repeated at least twice. Minor variations were found between experiments on different occasions. Results given are reliable to one significant figure only.

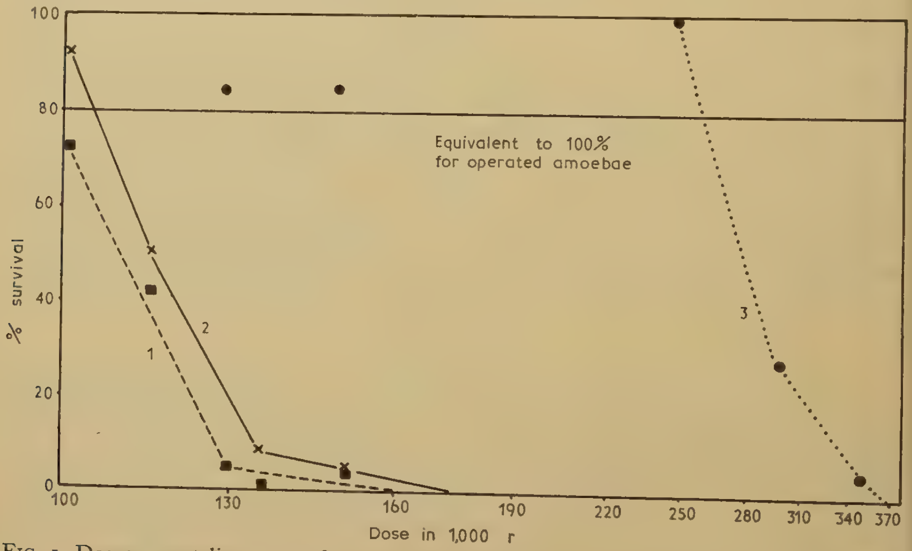

FIG. 1. Dosage mortality curves for *A. proteus* irradiated with 100,000 to 350,000 r X-rays: 1, $\overrightarrow{X_nN_c}$; 2, X_nX_c; 3, $\overrightarrow{N_nX_c}$. Each point represents approximately 25 amoebae.

Irradiated amoebae, X_nX_c. *A. proteus* were highly resistant to X-rays, doses of over 110,000 r being needed before lethal damage occurred (fig. 1, table 1). With doses of 120,000 r and 135,000 r only 50% and 10% respectively of the amoebae were able to form clones. Where damage was lethal, death generally occurred without division 1 to 7 weeks after treatment. Amoebae which had been lethally damaged were often partly rounded immediately after irradiation. Such amoebae became active and attached soon after irradiation and were able to feed and grow for several weeks. When damage was non-lethal, a short period of detachment and inability to feed followed irradiation with doses above 100,000 r, and a delay occurred before division.

Irradiated nuclei in normal cytoplasm $\overrightarrow{X_nN_c}$. When irradiated nuclei were put into normal cytoplasm the percentage survival was somewhat similar to that for X_nX_c amoebae (fig. 1, table 1), the slightly lower survival being due to a 10–20% loss from operational damage, e.g. at 110,000 r only 72% survival and at 120,000 r 41% survival. These amoebae became active and attached generally within a few hours of $X_n \rightarrow N_c$ transfer.

TABLE 1

Percentage of X_nX_c, $\overrightarrow{X_nN_c}$, *and* $\overrightarrow{N_nX_c}$ *amoebae able to divide, and percentage able to survive and form mass cultures after doses of X-rays from 110,000 to 350,000 r*

	Dose of X-rays in r	*Number of animals*	*% able to divide*	*% survival*
X_nX_c	110,000	20	95	95
	120,000	40	55	50
	130,000	20	40	30
	135,000	65	11	9
	150,000	140	7	5
	250,000	20	0	0
	300,000	100	0	0
	350,000	100	0	0
	Dose of X-rays in r	*Number of animals operated upon*	*% division of operated animals*	*% survival of operated animals*
$\overrightarrow{X_nN_c}$	110,000	22	86	72
	120,000	22	41	41
	130,000	25	8	4
	135,000	27	8	0
	150,000	47	4	4
$\overrightarrow{N_nX_c}$	130,000	23	87	87
	150,000	15	87	87
	250,000	21	100	100
	300,000	*		
	350,000	†		

* 135 amoebae were irradiated with a dose of 300,000 r; of these 100 became spherical immediately and died within 48 hours. Transfers attempted with these amoebae were all unsuccessful. Of the remaining 35 animals, transfers were made on 13, of which 11 divided and formed mass cultures.

† 200 amoebae were irradiated; 190 remained spherical until death and transfers made with these were all unsuccessful; of the 10 which partly recovered, 4 transfers were done which survived to form mass cultures.

Irradiated cytoplasm with normal nuclei $\overrightarrow{N_nX_c}$. Since transfers of $N_n \rightarrow X_c$ were largely unsuccessful if done from 0 to 18 hours after irradiation, owing to reversible cytoplasmic damage (section III), this section deals only with those $N_n \rightarrow X_c$ transfers done 18–48 hours after irradiation. After doses of X-rays up to 250,000 r, doses which were 100% lethal for X_nX_c and $\overrightarrow{X_nN_c}$

amoebae, over 80% of $\overrightarrow{N_n X_c}$ amoebae were able to form clones (fig. 1, table 1). Such amoebae remained floating for 1–2 days, after which they became active and attached. A delay occurred before the first division. Above 250,000 r irreversible cytoplasmic damage became apparent. Thus at 300,000 r all the amoebae became spherical after irradiation and 73% died within 48 hours. $N_n \rightarrow X_c$ transfers with these spherical amoebae were unsuccessful. Of the remaining 27%, which partly recovered their shape after 48 hours, over 80% formed mass cultures if given normal nuclei. With a dose of 350,000 r only 4% partly recovered, while 96% suffered from irreversible cytoplasmic damage.

With doses of 300,000 or 350,000 r, the animals were all spherical immediately after irradiation, and the great majority died within 48 hours, without any recovery. The death of these animals we consider to have been caused by cytoplasmic damage, for two reasons. First, when in the combination $\overrightarrow{X_n N_c}$ the nucleus is lethally damaged, death does not occur for a week or more. Second, the behaviour of cytoplasm from these heavily irradiated amoebae is similar to that of cytoplasm damaged in the absence of nuclei.

II. *Nuclear damage*

Transfers done within a short time of irradiation indicated that reversibly damaged cytoplasm could lethally damage nuclei (section III). Experiments were done to find whether any nuclear damage was directly caused by radiations or if all was due to contact of the nuclei with reversibly damaged cytoplasm. It was not possible to irradiate isolated nuclei, as nuclei which come in contact with the external media are not accepted again by the amoebae.

Direct damage could only be investigated by decreasing the time of contact between nuclei and cytoplasm after irradiation. For this experiment a 10-kV X-ray machine was used which gave a dose of 135,000–165,000 r in $5\frac{1}{2}$–6 minutes. The amoebae were irradiated on a plastic slide in a small drop of Chalkley's solution coated with liquid paraffin to prevent evaporation. Amoebae given doses of 135,000–165,000 r remained floating and died in 1–3 weeks.

'Quick transfers' were done as follows. Control amoebae were put into an operating chamber and enucleated. Twelve to fifteen normal amoebae were then put on a plastic slide and irradiated. As soon as irradiation ceased these amoebae were put into the operating chamber with the enucleated cytoplasms and $X_n \rightarrow N_c$ transfers done. The operation, from the *beginning* of irradiation to the *insertion* of the irradiated nucleus into normal cytoplasm, was timed. As only 5 to 6 transfers could be done within 30 minutes the whole procedure was repeated five times. Other $X_n \rightarrow N_c$ transfers done 2 and 40 hours after irradiation functioned as controls and $N_n \rightarrow X_c$ transfers were employed to check that cytoplasmic damage was not irreversible.

It is apparent from table 2 that all nuclei were lethally damaged within 15 minutes of the initial X-irradiation, and that longer contact with reversibly damaged cytoplasm did not increase the damage. The survival of a few transfers, $\overrightarrow{X_nN_c}$, where nuclei were removed in little more than 10 minutes from the beginning of irradiation, would, if significant, indicate that at least some of the nuclear damage was caused by contact with reversibly damaged cytoplasm.

TABLE 2

Survival of $\overrightarrow{X_nN_c}$ amoebae where contact between nuclei and cytoplasms from irradiation to transfer varied from 10 minutes to 40 hours

	Time from beginning of X-rays to end of transfer	*Number of animals*	*Number which divided*	*Number which formed mass cultures*	*Average length of life of animals which died* (days)
$\overrightarrow{X_nN_c}$	10–17 min.	11	3	2	13
	13–23 ,,	8	0	0	12
	17–24 ,,	10	0	0	13
	24–30 ,,	4	0	0	12
	2 hours	8	0	0	14
	40 ,,	8	0	0	15
X_nX_c		28	0	0	10
$\overrightarrow{N_nX_c}$	40 hours	5	5	5	

3. *Reversible cytoplasmic damage*

The occurrence of reversible cytoplasmic damage after irradiation with doses of over 100,000 r was indicated by (1) the increased 'stickiness' of the cytoplasm; (2) a tendency for the amoebae to burst when touched; (3) inability to attach, feed, or move actively; and (4) delay before first division of $\overrightarrow{N_nX_c}$ amoebae. Recovery from (1) and (2) occurred approximately 15–20 hours after irradiation, irrespective of dose up to 250,000 r; normal attachment and movement were generally resumed in 1–3 days, while division was delayed for as long as 10 days.

When doses above 250,000 r were given reversible damage became more prominent, all amoebae becoming spherical. Recovery from such damage took up to 9 days, depending on the dose, and all transfers attempted before recovery were unsuccessful.

X-rays given with the 10-mA X-ray machine increased reversible cytoplasmic damage as observed by (1) the clumping of cytoplasmic granules in the centre of the amoebae 10–30 minutes after irradiation, and (2) the enormous increase in size of the contractile vacuole approximately 1 hour after irradiation. These symptoms disappeared in a few hours if the amoebae were left undisturbed, but if poked or moved about the animals generally burst.

That reversible damage had occurred with doses between 100,000–300,000 r was confirmed by the ability of such cytoplasm to damage control nuclei. This was shown by (*a*) the swelling of control nuclei when put into cytoplasm within a few hours of irradiation, and (*b*) the small percentage of $N_n \rightarrow X_c$ transfers, done within 18 hours of irradiation, that were able to form mass cultures. The latter is illustrated in table 3, where in two experiments only 36% and 50% transfers done 3–8 hours after irradiation formed mass cultures, whereas over 80% of transfers done 24 hours after irradiation survived. In

TABLE 3

Increased survival of $N_n \rightarrow X_c$ *transfers made 24 hours after irradiation over* $N_n \rightarrow X_c$ *transfers made 3–8 hours after irradiation*

Dose of X-rays in r	*Number of transfers*	*Time of transfer after irradiation*	*% survival*
		(hours)	
150,000	25	3–8	36
150,000	28	3–8	50
130,000	23	22–26	87
150,000	15	22–26	87
250,000	21	20–24	100

TABLE 4

*Delays in division following irradiation**

	Dose in r	*Time between irradiation and first division*	*Time between irradiation and second division*
		(days)	(days)
X_nX_c (200–250 amoebae)	10,000	2	6
	20,000	3	7
	50,000†	4	8
	70,000†	6	9
	90,000†	8	12
	110,000	8	11
	120,000	12	16
	130,000	15	18
	135,000	14	16
	150,000	12	18
$\overrightarrow{N_nX_c}$ (150–200 amoebae)	120,000	9	11
	130,000	10	14
	135,000	7·5	9
	150,000	10	12
	250,000	7	9
	300,000	9	10

* Since the results obtained for $X_n\overrightarrow{N}_c$ amoebae are based on only a small number of divisions, they have not been included in this table.

† X-rays given with a 220 kVp, 15 mA machine, distance from source 9·3 cm.

approximately half of the unsuccessful transfers death, which occurred within

24 hours, was due to damage during transfer caused by 'stickiness' and bursting. In the remaining half death was due to damage done to control nuclei by the reversibly damaged cytoplasm and occurred 1–7 weeks after transfer.

IV. *The effect of radiation on division*

Irreversibly damaged nuclei or cytoplasms seldom divided, so that almost all X_nX_c, $\overrightarrow{X_nN_c}$, and $\overrightarrow{N_nX_c}$ amoebae which divided were capable of forming mass cultures. Where damage was not lethal, the first division after irradiation was delayed with all doses above 100,000 r (table 4). This delay in X_nX_c amoebae increased with doses from 50,000 to 120,000 r, above which there was no further increase. With doses of 120,000 to 150,000 r, the first division of X_nX_c and $\overrightarrow{X_nN_c}$ amoebae occurred approximately 13 days after irradiation, slight variations being due to differences in food and temperature during the experiments. With doses of 120,000 to 300,000 r the first division of $\overrightarrow{N_nX_c}$ amoebae occurred approximately 8 days after irradiation.

The second division of X_nX_c and $\overrightarrow{X_nN_c}$ amoebae was slightly delayed, but with few exceptions the second division in $\overrightarrow{N_nX_c}$ amoebae showed no delay.

DISCUSSION

Doses of X-rays needed to cause lethal damage in amoebae were much greater than those required for most other cells, though the effects of irradiation were similar to effects observed in other cells. Workers using X-rays have shown that such radiations are able to cause many types of damage, such as chromosomal changes, changes in nucleoli, mutations, protein denaturation, depolymerization of long molecules (e.g. desoxyribonucleic acid), inactivation of enzymes, gel to sol changes in the spindle, and cytoplasmic changes such as vacuolization, degeneration of plastids and mitochondria, and changes in pH and viscosity (e.g. Lea, 1947; Spear, 1946; Gray, 1946; Sparrow and Rubin, 1951). Some of these changes require very low doses of X-rays, while others require larger, but all have been obtained with doses of 100,000 r or lower. Since doses of X-rays lethal to *A. proteus* were above 100,000 r, radiation may have caused any of the above effects.

Death of *A. proteus* after less than 250,000 r is usually due to irreversible nuclear damage. The lethal dosage for cytoplasm is about twice that for nuclei. Irradiated cytoplasm is, however, reversibly damaged to an observable extent by doses of X-rays between 120,000 and 250,000 r. Recovery from most of this damage is completed in 24 hours. Such reversibly damaged cytoplasm is able to damage normal nuclei lethally. Thus nuclear damage appears to be a combination of direct injury by the X-rays and indirect injury by contact with reversibly damaged cytoplasm. It is already maximal by 15 minutes from the beginning of irradiation. Unfortunately, since nuclei cannot be irradiated apart from cytoplasm, it has not been possible to separate the direct and indirect damage to nuclei. Although we have been unable to prove that nuclei of *A. proteus* are directly damaged by X-rays, the fact that a nucleus, which has not itself been irradiated, will be lethally damaged by exposure to irradiated cytoplasm which is not itself lethally damaged, is a point of major interest. It shows, for example, that it would be futile to attempt to analyse nuclear damage in terms of the 'target theory'. Indeed, one of the clearest results of our study of the action of 'nitrogen mustard' (Ord and Danielli, 1956; Ord, 1956), and of this present study of the action of X-rays, by the use of nuclear transfer, is that direct action upon cytoplasms is of major importance. This suggests that the same may be true of the action of these agents upon other cells, and that the extent of direct action upon nuclei has been exaggerated. A. Glücksmann has frequently pointed out that many, if not all, of these nuclear cytological abnormalities produced by radiations and 'mustards' are found in tissue cultures under abnormal conditions of nutrition, which suggests that cytoplasmic abnormalities may result in nuclear damage. Observations of this nature go back at least as early as Strangeways (1924). Lasnitski (1943) gives the percentage of abnormal mitoses in normal cultures as $7 \pm 1{\cdot}1\%$.

The nucleus of *A. proteus* contains a large number of extremely small chromosomes and is thought to be many times polyploid. If this is so, then chances of lethal damage by structural chromosomal changes or mutations would be decreased; for if each gene has many duplicates, then one direct chromosome hit would have little observable effect. It is notable that the large amoeba *Pelomyxa carolinensis* (Daniels, 1951, 1952), which contains several hundred smaller nuclei, has a resistance to X-rays similar to that of *A. proteus*.

The great resistance of Protozoa to X-rays may be due to resistance of either nucleus or cytoplasm or both. Many substances, such as SH groups, are effective in protecting cells from radiations (e.g. Sparrow, 1951). No comparisons of the occurrence of such substances have been made between Protozoa and other cells more sensitive to X-rays. It is possible that the cytoplasms of some Protozoa may contain large quantities of protective substances, while polyploid nuclei may protect the animals against structural chromosomal damage and mutations.

ACKNOWLEDGEMENTS

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The Site of Damage in Amoebae exposed to Low Concentrations of Methyl di-(β-Chloroethyl)-Amine (a 'Nitrogen Mustard')

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SUMMARY

Amoeba proteus was exposed to a low concentration of methyl di-(β-chloroethyl) amine (MBA, a 'nitrogen mustard'). Subsequent nuclear transfer was used to study the relative sensitivities of nucleus and cytoplasm to MBA. Nuclei were found to be 10 times as sensitive as cytoplasm. Exposure to MBA caused a short delay before the first division of the nucleus and a longer delay before the second division, the delay before the first division being more marked with exposure to LD_{100} or higher concentrations. The first cytoplasmic division was delayed after treatment but subsequent division showed little change. MBA caused many abnormalities in division. When cytoplasm was injured the nuclei became swollen or, with less severe damage, divided without subsequent cytoplasmic division. When only nuclear damage was present, such abnormalities as incomplete prophase, non-nucleate division, uneven division, and cytoplasmic fragmentation occurred.

PREVIOUS work (Ord and Danielli, 1956*a*) with LD_{100} concentrations of the 'nitrogen mustard', methyl di-(β-chloroethyl)-amine (MBA) and the technique of nuclear transfer, showed that the cytoplasm of *A. proteus* was directly damaged by the drug, while the nuclei were probably damaged directly but could also be damaged indirectly by contact with injured cytoplasm. The experiments reported in this paper were devised to compare the sensitivities of nuclei, cytoplasm, and nuclei plus cytoplasm to this drug, and studies were also made of delays and abnormalities which occurred during division.

METHODS

Culture methods and the technique of nuclear transfers were similar to those described by Lorch and Danielli (1953). The method of exposing amoebae to the drug has been described in Ord and Danielli (1956*a*). Duration of exposure was 15 minutes in all cases. The concentrations of MBA used ranged from LD_0 to LD_{100}, from 0·0000075 to 0·0025%.

Abnormalities in the division of amoebae treated with MBA have been studied in living animals. The actual nuclear divisions were not observed, but the products of division generally enabled one to determine the course of events.

Nomenclature is according to the scheme of Lorch and Danielli (1953). Thus the transfer of a MBA nucleus to normal cytoplasm would be $MBA_n \rightarrow N_c$, the resultant animal being $\overrightarrow{MBA_n N_c}$; transfer of a normal nucleus to cytoplasm treated with MBA would be $N_n \rightarrow MBA_c$, the resultant animal being $\overrightarrow{N_n MBA_c}$; treated unoperated animals would be $MBA_n MBA_c$.

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Results

A. *Nuclear and cytoplasmic sensitivities*

In the following work all experiments were repeated at least twice. Results are reliable to one significant figure only, but not to two, as minor variations were found between experiments on different occasions.

Amoebae treated with MBA, $\mathrm{MBA}_n\mathrm{MBA}_c$. Concentrations below 0·0000075 had no observable effects on *A. proteus*. Between 0·00001 and 0·0025% the percentage killed ranged from 10 to 98%, the LD_{50} concentration being approximately 0·0001%. These results varied slightly with temperature, which was not regulated. Results given were at temperatures of 18–24° C.

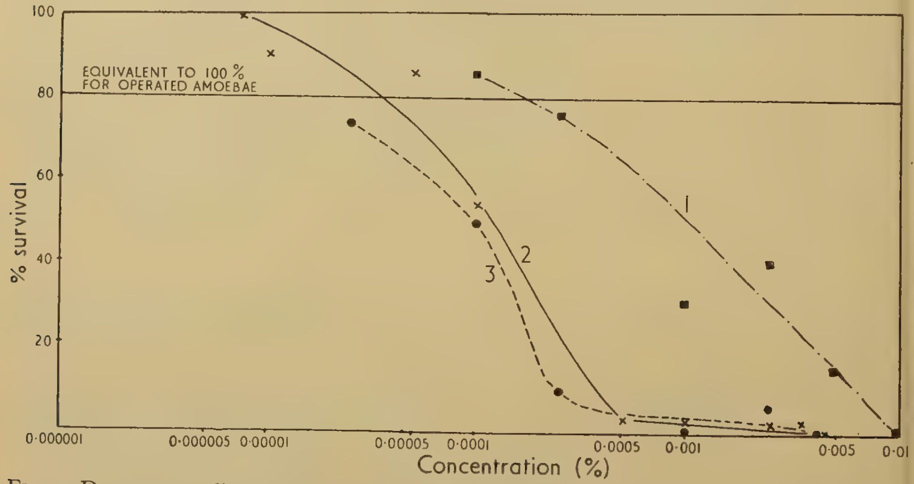


Fig. 1. Dosage-mortality curves of *A. proteus* treated with 0·0000075 to 0·01% MBA. Each point represents 10–30 amoebae. Curve 1, $\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$ amoebae ■—■; curve 2, $\mathrm{MBA}_n\mathrm{MBA}_c$ amoebae ×—×; curve 3, $\mathrm{MBA}_n\mathrm{N}_c$ amoebae ●—●.

Treated nuclei in normal cytoplasm, $\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c}$. When nuclei treated with 0·0000075 to 0·0025% MBA were transferred to normal cytoplasm, survival was similar to that of $\mathrm{MBA}_n\mathrm{MBA}_c$ amoebae (fig. 1, table 1). Concentrations of MBA from 0·00001 to 0·0025% were 10 to 95% lethal, the LD_{50} concentration being approximately 0·0001%.

Normal nuclei in treated cytoplasm, $\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$. The greater resistance of the cytoplasm to MBA, found in previous experiments (Ord and Danielli, 1956*a*), was confirmed when lower concentrations of the drug were used. Concentrations of MBA from 0·00025 to 0·0025% were 10 to 60% lethal. Concentrations of 0·0001 and lower caused no irreversible damage.

The LD_{50} concentration of nitrogen mustard for $\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$ amoebae was approximately 10 times greater than that for $\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c}$ or $\mathrm{MBA}_n\mathrm{MBA}_c$ amoebae; i.e. the nuclei were about 10 times more sensitive than the cytoplasms.

B. *Delays in division*

Exposure of amoebae to MBA caused a delay of both the first and the second divisions after treatment (table 2). This delay was found in $\mathrm{MBA}_n\mathrm{MBA}_c$, $\overrightarrow{\mathrm{MBA}_n}\mathrm{N}_c$, and $\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$ amoebae. Transfers were done within 24 hours of treatment with the drug. Control amoebae generally divided every two days.

TABLE 1

Percentage division and percentage survival of $\mathrm{MBA}_n\mathrm{MBA}_c$, $\overrightarrow{\mathrm{MBA}_n}\mathrm{N}_c$ *and* $\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$ *amoebae treated with* 0·0000075 *to* 0·0025% *MBA*

Type	*Concentration of MBA*	*Number of amoebae*	*% division*	*% survival formed clones of 20–30 amoebae*
$\mathrm{MBA}_n\mathrm{MBA}_c$	0·0000075	20	100	100
	0·00001	20	90	90
	0·00005	65	90	86
	0·0001	95	85	54
	0·0005	60	82	3
	0·001	180	56	3
	0·0025	120	42	2
$\overrightarrow{\mathrm{MBA}_n}\mathrm{N}_c$	0·000025	25	84	76
	0·0001	22	60	50
	0·00025	20	65	10
	0·001	10	40	0
	0·0025	15	55	7
$\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$	0·0001	20	85	85
	0·00025	20	75	75
	0·001	20	30	30
	0·0025	16	40	40

With the lowest concentration of MBA used the times for the first and the second divisions of $\mathrm{MBA}_n\mathrm{MBA}_c$ amoebae after exposure to the drug were similar to those of controls. This concentration was not lethal. With a concentration of 0·00001% there was a slight delay before the first division after exposure, but no delay between the first and second divisions. With concentrations from 0·00001 to 0·0005% ($\mathrm{LD}_{10\cdot 90}$) the first division after exposure was only slightly delayed, but the delay before the second division increased with concentration. With concentrations of MBA above 0·001% ($\mathrm{LD}_{90\cdot 100}$) generally only one division occurred, and this first division after exposure, which had shown only a slight and consistent delay of 1–3 days with lower concentrations, was considerably delayed; thus with 0·001, 0·0025, and 0·005% MBA the first division occurred 6, 7, and 12 days after treatment. $\mathrm{MBA}_n\mathrm{MBA}_c$ amoebae seldom divided at concentrations above 0·005%.

Times between exposure to the drug and the first division for both $\overrightarrow{\mathrm{MBA}_n}\mathrm{N}_c$ and $\mathrm{N}_n\overrightarrow{\mathrm{MBA}_c}$ amoebae showed a delay similar to $\mathrm{MBA}_n\mathrm{MBA}_c$ amoebae, with an added delay of 2–4 days due to operational damage. The time between exposure and the second division of $\overrightarrow{MBA_n}N_c$ amoebae was similar to that for MBA_nMBA_c amoebae, but the second division of $N_n\overrightarrow{MBA_c}$ amoebae was delayed only 1–2 days.

TABLE 2

Time of first and second divisions after treatment with MBA for MBA_nMBA_c, $\overrightarrow{MBA_n}N_c$, and $N_n\overrightarrow{MBA_c}$ amoebae treated with 0·0000075 to 0·005% MBA

Type	Concentration of nitrogen mustard %	Time between treatment and first division	Time between treatment and second division	% survival
		(days)	(days)	
MBA_nMBA_c	0·0000075	2	4	100
	0·00001	3	5	90
	0·000025	3	7	75
	0·00005	5	8	86
	0·0001	4	11	54
	0·00025	5·5	17	5
	0·0005	4·5	13	3
	0·001	6		3
	0·0025	7		2
	0·0035	9		0·5
	0·005	12		0·3
$\overrightarrow{MBA_n}N_c$	0·000025	6	7	76
	0·0001	5	11	50
	0·00025	9	16	10
	0·001	11		0
	0·0025	11		7
	0·0035	12		10
	0·0045	10		2
	0·005	9		1·5
$N_n\overrightarrow{MBA_c}$	0·0001	6	11	85
	0·00025	7	10	75
	0·001	7	11	30
	0·0025	11	14	40
	0·0035	11	14	32
	0·0045	12	14	18
	0·005	13	14	12
N_nN_c	Nil	2	4	100

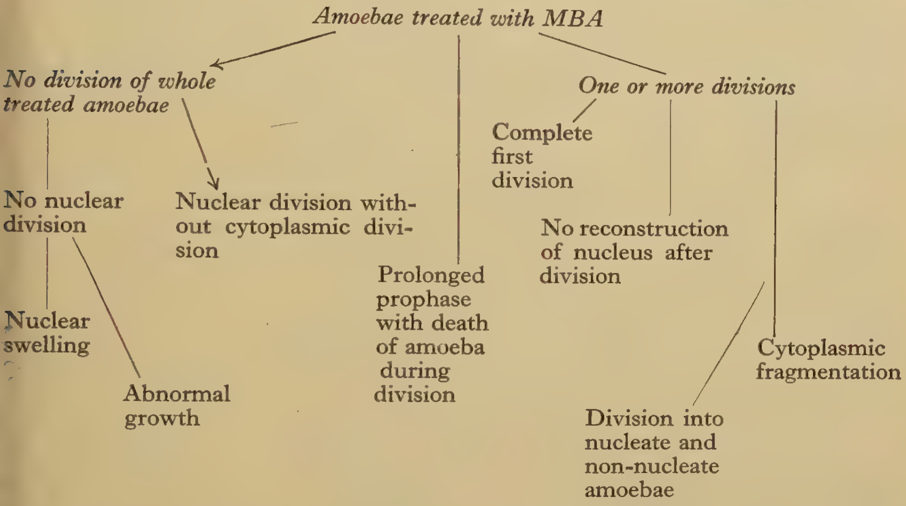
Thus exposure to MBA delayed cytoplasmic division even at concentrations where damage was reversible. This damage was probably repaired before any division took place, as there was little delay for subsequent divisions. Exposure of nuclei to the MBA slightly delayed first divisions and caused a considerable delay of second divisions, nuclei being able to divide before damage had been repaired. It is therefore probable that with MBA_nMBA_c amoebae the delay which occurred between the first and the second divisions after exposure was due in most cases to nuclear damage.

C. *Abnormalities in division*

The nucleus of *Amoeba proteus* is flattened. It is about 40μ in greatest diameter. During binary fission it undergoes mitosis. There is a large number of minute chromosomes. Living amoebae were generally studied.

Most abnormalities observed were of MBA_nMBA_c or $\overrightarrow{\text{MBA}_n}\text{N}_c$ amoebae, though occasionally abnormalities occurred during the division of $\text{N}_n\overrightarrow{\text{MBA}_c}$ amoebae. It must also be noted that many of these abnormalities were occasionally found in very poor control cultures where food was scarce or moulds abundant.

Abnormalities were of two types, as is shown diagramatically below.


Amoebae treated with MBA
No division of whole treated amoebae
One or more divisions
No nuclear division
Nuclear division without cytoplasmic division
Nuclear swelling
Abnormal growth
Prolonged prophase with death of amoeba during division
Complete first division
No reconstruction of nucleus after division
Division into nucleate and non-nucleate amoebae
Cytoplasmic fragmentation

Swelling of nuclei occurred where the cytoplasm was extensively damaged by the MBA in both MBA_nMBA_c and $\text{N}_n\overrightarrow{\text{MBA}_c}$ amoebae. Such nuclei failed to divide. When pricked with a sharp needle they collapsed with the escape of fluid, leaving only a thin nuclear membrane. This remnant, if transferred to new cytoplasm, again became swollen. Nuclei of normal amoebae are a solid gel and can be punctured without the occurrence of any visible change.

When damage to the cytoplasm was less severe, nuclear division occurred but was not followed by cytoplasmic division. Both nuclei and cytoplasms of such animals continued to grow for 2 or 3 weeks, reaching abnormal sizes. Because of the irregular shapes of both nuclei and cytoplasms, measurements were difficult; but growth was roughly twice the diameter for both nuclei and cytoplasms. A small percentage of the animals had four or more nuclei.

With lower concentrations of MBA, where the cytoplasm was relatively uninjured but the nuclei were either reversibly or irreversibly injured, the following types of abnormalities occurred.

(1) Incomplete division, during which the amoebae formed a division

sphere which burst after a prophase lasting many hours. Prophase in controls lasted 10–15 minutes.

(2) Complete first divisions giving two daughter amoebae, often very uneven in size, which appeared normal initially but were unable to continue dividing.

(3) Divisions giving one or more non-nucleate amoebae. The nucleate amoebae from such divisions were sometimes able to continue dividing.

(4) Cytoplasmic fragmentation where one or more cytoplasmic fragments were shed without the prior formation of a division sphere.

(5) The formation of a division sphere followed by a separation into two daughter amoebae, but with no reconstruction of the nucleus in either animal.

DISCUSSION

The results obtained from this work show the relative sensitivities of the nucleus and cytoplasm of *Amoeba proteus* to methyl di-(β-chloroethyl) amine and enable a comparison to be made with results obtained with X-rays (Ord and Danielli, 1956*b*). Nuclei were more sensitive than cytoplasm to both the drug and X-rays, the nuclear to cytoplasmic sensitivity ratios being approximately 1:10 and 1:2·5 respectively. The steep slope of the dosage mortality curves for nuclear and cytoplasmic injury after irradiation, and the gradual slope of nuclear and cytoplasmic injury curves after treatment with MBA indicate that there was a clearer separation of nuclear and cytoplasmic damage with X-rays than with the MBA. Thus, a concentration of MBA which killed 90% of the nuclei lethally damaged 25% of the cytoplasms, whereas a dose of X-rays sufficient to kill all nuclei caused no irreversible damage to cytoplasms. Approximations of the LD_{10}, LD_{50}, and LD_{90} with the MBA treatment and the irradiation for nuclei and cytoplasms are given in Table 3.

TABLE 3

Lethality of methyl di-(β-chloroethyl) amine (MBA)

	Nucleus	*Cytoplasm*	*LD nucleus/LD cytoplasm*
LD_{10} . . .	0·00002%	0·0002%	0·1
LD_{50} . . .	0·00015%	0·001–0·0025%	0·06–0·15
LD_{90} . . .	0·0005–0·0025%	0·005%	0·1–0·5
	Lethality of X-rays		
	Nucleus	*Cytoplasm*	*LD nucleus/ LD Cytoplasm*
LD_{10} . . .	110,000 r	285,000 r	0·38
LD_{50} . . .	120,000 r	290,000 r	0·41
LD_{90} . . .	135,000 r	340,000 r	0·39

Behaviour of amoebae after treatment indicated that both MBA and X-rays caused reversible cytoplasmic damage. This reversible damage was very pronounced after irradiation, and such cytoplasm was able to damage normall nuclei lethally. Cytoplasm lethally damaged by MBA or X-rays could also lethally damage normal nuclei.

Treatment with concentrations of MBA which damaged nuclei but caused no irreversible damage to cytoplasm produced a short delay before first division and a long delay between first and second division. Similar results were obtained by Daniels (1952) with *Pelomyxa carolenesis*. Daniels suggested that the 'nitrogen mustard' combined reversibly with an enzyme which synthesized a substance necessary for division. If enough end-product had already been formed, one division would take place with little delay; before a second division could occur the block must be overcome. Since this delay between first and second division was marked in $\mathrm{MBA}_n\mathrm{MBA}_c$ and $\overrightarrow{\mathrm{MBA}_n\mathrm{N}_c}$, but not in $\overrightarrow{\mathrm{N}_n\mathrm{MBA}_c}$, amoebae, any such block must occur in the nucleus. When amoebae were treated with lethal concentrations soon after a division, there was seldom any division following the treatment in either *Pelomyxa* (Daniels, 1952) or *A. proteus.*

After irradiation there was seldom any division if nuclear damage was lethal, but sublethal doses of X-rays produced a delay between irradiation and the first division. Little delay occurred for subsequent divisions.

When cytoplasm was reversibly damaged by either X-rays or MBA, a delay was produced between treatment and first division, but subsequent divisions were normal. No division occurred when cytoplasm was lethally damaged by either the drug or radiations.

The many abnormal types of division which occurred after treatment with MBA have been noted in section C. These include swelling of nuclei, nuclear division without cytoplasmic division following cytoplasmic injuries, prolonged prophase, no nuclear reconstruction after division, uneven divisions, formation of anucleate amoebae, and cytoplasmic fragmentation following nuclear injury. Far fewer abnormalities occurred after treatment with X-rays, since irradiated amoebae seldom divided when damage was lethal.

I should like to thank Professor J. F. Danielli and Dr. L. G. Bell for their help and advice. I am indebted to the British Empire Cancer Campaign for financial aid and to the Royal Society and the Rockefeller Foundation for assistance with apparatus.

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The Fate of 'Thorotrast' (Thorium Dioxide) injected into the Dorsal Lymph Sac of the Frog, *Rana temporaria*

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With 5 plates (figs. 1–5)

SUMMARY

Thorotrast (a colloidal suspension of thorium dioxide) injected into the dorsal lymph sac of the common frog, *Rana temporaria*, can be shown by radiological methods to pass through the anterior lymph hearts and so into the blood system. From the blood the thorotrast is removed, as in mammals, by cells of the reticulo-endothelial system and by macrophages, the sites of greatest activity being the liver, spleen, and bone-marrow. Whereas in mammals almost all uptake of such colloidal substances takes place in these organs, in the frog there is considerable macrophage activity elsewhere, notably in the submucosa of the alimentary canal. Our results suggest that macrophages which ingest thorium particles in the wall of the alimentary canal migrate through the blood system to the liver where the thorium is ultimately deposited. This may be correlated with the possession by the frog of a macrophage and reticulo-endothelial system less highly organized than that possessed by mammals.

INTRODUCTION

WHEN it is desired to ascertain the reactions of frogs to various drugs and other materials, the dorsal lymph sac is frequently utilized as a site of injection. The use of the dorsal lymph sac for this purpose is favourable on account of the very rapid lymph circulation, which has been investigated in some detail in *Rana pipiens* by Conklin (1930), who found that this lymph circulation dealt with amounts of fluid which, when compared with the body weight of the frog and the total blood-volume, were indeed considerable.

It occurred to us that this circulation of lymph might be made visible by the injection into the dorsal lymph sac of the contrast medium 'thorotrast' (a colloidal suspension miscible with blood, containing 24–26 per cent. of thorium dioxide by volume), followed by serial radiographs. As our results show, this has been possible and the movement of the thorotrast from the lymph sac to the blood-stream has been demonstrated radiologically. Radt (1930) and Volicer (1931) described the use of thorotrast in hepato-lienography, i.e. the radiological investigation of the liver and spleen. As these authors pointed out, the principle of this technique is that colloidal particles injected into the blood-stream are removed by phagocytic activities of the reticulo-endothelial system and that if such colloidal particles are radio-opaque they produce substantial shadows on an X-ray plate. Such shadows not only show the extent of the organ in which the substance has accumulated but also give information as to the activity of the tissues responsible for its uptake. Although on its introduction this technique of hepato-lienography

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was claimed to be harmless to the patient, much controversy has develope over the possible effects of the prolonged storage in the human body of substance which is radio-active. For, once taken up by the body, there is neve any considerable elimination of the thorotrast.

Research on the uptake and fate of thorotrast appears to have been confine to mammals including dogs, rats, mice, guinea-pigs, and rabbits (Irwin, 1932 Tripoli, 1934). There is also information as to the fate of the thorotrast i man after a considerable number of years (see Cassel, Ruffin, Reeves, an Stoddard, 1951). As the result of our injections of thorotrast into the dorsa lymph sac of frogs, we have been able to observe not only its transfer to th blood but its removal from the blood to the liver and spleen. This process o removal seems to us not altogether simple, and we have come upon feature of the process which appear to merit description as illustrating the propertie of the reticulo-endothelial system.

EXPERIMENTS

Procedure

Specimens of the common frog, *Rana temporaria*, obtained from dealer were used in these experiments. The majority weighed about 20 g, but a few were somewhat heavier. They were kept at room temperature throughout th experiments. The thorotrast was injected with fully aseptic precautions int the dorsal lymph sac. The amount varied. In experiments designed to trac the path of the thorotrast from the lymph sac into the circulation 0·5 c.c. was used, but in the majority of experiments to investigate the ultimate fate of th thorotrast the quantity was reduced to 0·1 c.c. Even this is greatly in excess of the dose of 0·8 c.c. per kilogram of body weight suggested for man an other mammals by Tripoli (1934).

The frogs were radiographed at various intervals after injection. Th radiographic factors used were standardized at 100 mA 35 kv 0·4 second at a focus-film distance of 100 cm. The exposures were made on envelope packed 'Ilfex' X-ray film (for use without intensifying screens) and th development time was 4 minutes at about 24° C.

The frogs were allowed to survive until such time as it was desired to compare the radiographs with sections of the various organs. During this tim the frogs were fed regularly on suitable material such as earthworms.

When the frogs were killed, the organs to be investigated were fixed in Zenker-formaldehyde solution, and after appropriate treatment the resultin sections were stained in Ehrlich's haematoxylin and counterstained in eosin

RESULTS

The lymphatic pathway

To show what happens when thorotrast is injected into the dorsal lymp sac of a frog a rather heavy injection (0·5 c.c.) was used. The results of such an experiment are shown in fig. 1.

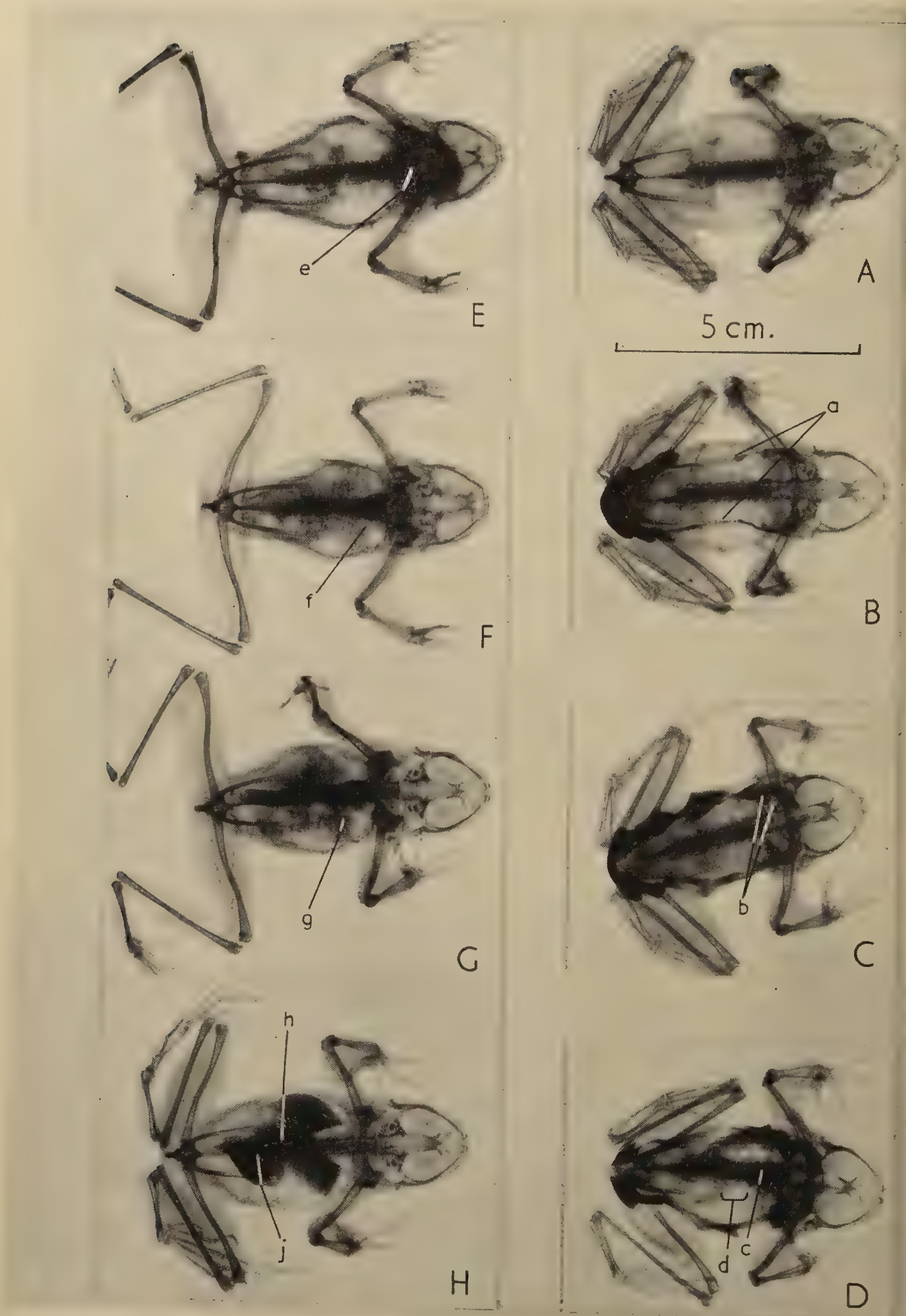

Fig. 1

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The normal frog before injection is shown in fig. 1, A and the state immediately after injection in B. Although the injection was made well anterior to the sacral prominence the thorotrast, presumably because it is heavy, has sunk to the lower parts of the sac, and already, in the few seconds that have elapsed between injection and the making of the X-ray, two streams of thorotrast have begun to move forwards along the margins of the dorsal lymph sac (*a* in B). In C (15 minutes after injection) a considerable forward movement of the thorotrast has taken place, some of it no doubt due to movements of the frog, and shadows have appeared (*b*) in the region of the anterior venae cavae, to which the anterior pair of lymph hearts are connected by the vertebral veins. In D (45 minutes after injection) the heart is giving a pronounced shadow (*c*) as are the vessels in the lungs (*d*). In addition there are blood-vessels clearly shown in the right leg. E (4 hours after injection) shows that there is little, if any, thorotrast left in the lymph sac. In the region of the heart and sternum (*e* in E) a dense shadow is to be seen; the lung fields are well filled and the blood-vessels of the legs are very conspicuous. Shadows which have begun to appear may be cast by the spleen and portions of the liver, but this is uncertain.

F shows the frog 24 hours after injection. The blood system is still visible in the fore limbs and more clearly in the hind limbs. The liver has started to cast its unmistakable shadows (*f*) and there are other shadows in the region of the intestine.

In G (48 hours after injection) the blood system is still marked and the liver is becoming well defined (*g*).

Finally, H shows the frog 8 days after the injection. The thorotrast has disappeared from the circulation. Dense shadows are cast by the liver and spleen (*h*) and there is also an accumulation of radio-opaque material in the intestine (*j*). Although this frog was kept for another 7 days there was no significant change in the shadows. When the frog was killed the shadow in the

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FIG. 1 (plate). A, radiograph of normal frog.

B–H, radiographs of the same frog at various times after injection of 0·5 c.c. thorotrast into the dorsal lymph sac.

B, immediately after injection.
C, 15 minutes after injection.
D, 45 minutes after injection.
E, 4 hours after injection.
F, 24 hours after injection.
G, 48 hours after injection.
H, 8 days after injection.
a, thorotrast passing forwards along the lateral walls of the dorsal lymph sac.
b, shadows caused by thorotrast in the anterior venae cavae.
c, shadow of heart.
d, shadows of vessels of right lung.
e, region of heart and sternum.
f, shadow of right lobe of liver.
g, shadow of right lobe of liver, more intense.
h, shadow of spleen.
j, shadow caused by mass of radio-opaque material in intestine.

(For full description see text.)

intestine was found to be caused by a large brown mass, presumably thor trast, which had obtained access to the intestine. This is not to be regarded normal. An apparently similar frog injected with the same quantity of thor trast at the same time did not show any shadow in the lumen of the alimenta canal during a similar period. This result was probably caused by the injectio of too large a quantity of thorotrast to be dealt with by the normal mechanism and so in subsequent experiments the amount injected was considerab reduced, and this circumstance was not met with again. (This matter will b commented upon again later.)

Thus thorotrast injected into the dorsal lymph sac is rapidly removed b the action of the anterior pair of lymph hearts, which, by their contraction pass the lymph into the vertebral vein on each side of the body and so throug the anterior venae cavae into the heart. We were unable to detect in th radiographs the return of any of the thorotrast from the lymph sac by wa of the posterior lymph hearts and iliac veins.

The removal of the thorotrast from the blood-stream

In the account just given of the lymphatic pathway it is to be noted tha the events following injection of the thorotrast into the dorsal lymph sac ar not clearly separable in time but merge into each other; thus the shadows o the liver and spleen begin to become apparent while thorotrast is still in th blood-stream. The process of removal from the blood cannot be separate off, for presumably it begins as soon as thorotrast enters the blood but is no detectable radiologically until it has been taking place for some time.

For the investigation of this process it was found desirable to make a smalle injection than 0·5 c.c., and in an injection with 0·1 c.c. of thorotrast the dilutio is such that the blood-vessels show only faintly. The vessels of the legs ca be discerned in a radiograph taken 3 hours 45 minutes after injection. A radio

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FIG. 2 (plate). A–D, radiographs of a frog injected with 0·1 c.c. thorotrast.
A, 27 hours after injection.
B, 2 days after injection.
C, 3 days after injection.
D, 16 days after injection.
Note the gradual intensification of the shadow of the liver *l.*, and the considerable variation o hadows in the region marked *x*. *s*, spleen.
E–G, radiographs of a frog injected with 0·1 c.c. thorotrast.
E, 4 days after injection.
F, 8 days after injection.
G, 36 days after injection.
Again note variation of shadows in region marked *x*.
H, radiographs of a frog injected with 0·1 c.c. thorotrast 28 hours previously. Note ver marked spleen shadow *s*.
I–L, radiographs of a frog injected with 0·1 c.c. thorotrast.
I, after 2 days.
J, after 7 days.
K, after 24 days.
L, after 133 days.
Note the gradual accumulation of thorotrast in the liver and the gradual diminution in the size of the spleen.

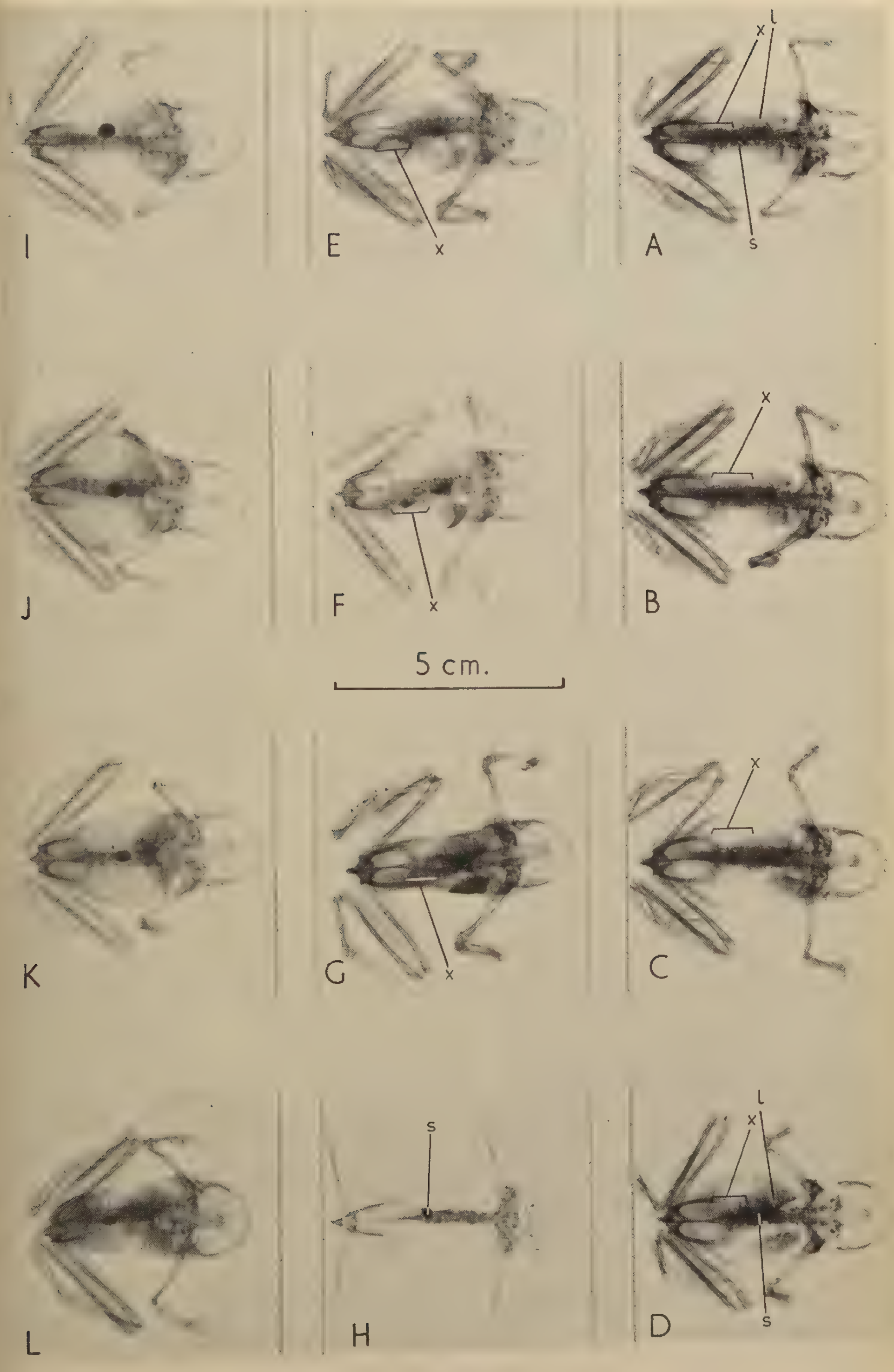

FIG. 2

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graph taken 24 hours after injection shows the shadow of the liver, and a slightly later radiograph (3 hours later still) is reproduced in fig. 2, A. Fig. 2, B shows a radiograph made 2 days after injection; C, 3 days after, and D, 16 days after injection. The main point of interest is that during this time the liver shadow becomes distinctly more intense. A point we noted was that shadows in the area marked *x* in A–D showed some variation, and the explanation that occurred to us was that thorotrast which had been removed in some way from the blood circulation was being transferred to the liver through the portal system. Thus it might be that the final site of the thorotrast was not necessarily that at which it left the blood-stream.

Similar changes in the distribution of thorotrast after its disappearance from the blood-stream were found in other experiments. Fig. 2, E–G shows a frog injected with 0·1 c.c. thorotrast 4, 8, and 36 days after injection. There is considerable difference between these radiographs in the distribution of the thorotrast.

There are various shadows in the region of the intestine which have been shown, by removing the alimentary canal and radiographing it outside the body, to be caused by thorotrast in the spleen and also in neighbouring tissues, including the wall of the alimentary canal itself. This is well shown in fig. 4, E and F.

The distribution of thorotrast between liver and spleen at an early stage in its removal from the blood system is by no means constant. In fig. 2, A (29 hours after injection) there is a well-defined liver shadow. In another frog similarly injected (fig. 2, H, 28 hours after injection) the spleen shows up well and the liver hardly at all. (The liver shows up slightly in the original X-ray, but in printing to obtain contrast between the spleen and the vertebral column the liver ceases to be apparent.)

There is therefore some degree of variation in what may take place when the thorotrast is removed from the blood. Such variation may be correlated with variation in the activity of the cells of the reticulo-endothelial system. In rats the activities of these cells have been shown by Gordon and Katsh (1952) to be associated with the nutritional state of the animal and the activity of its adrenal glands. Some of the variation in the uptake of the thorotrast which we have described may have been due to such causes.

Some of the movements of the thorotrast after it has left the blood are of considerable duration. For example, in fig. 2, I–L are shown the conditions in the same frog 2, 7, 24, and 133 days after injection respectively. Accumulation of thorotrast in the liver and the gradual diminution in the size of the spleen is readily seen.

These relatively slow movements of thorotrast led us to inquire into the actual method by which it leaves the blood and accumulates in the liver and spleen. In order to do this, sections were cut of organs from injected animals and an attempt made to correlate the micro-anatomy with the radiological anatomy.

The particles of thorotrast as injected are not visible under the microscope, but in cells it appears as highly refractile particles. How this comes about is not at present clear. At some stage flocculation of the colloidal particles must take place. According to Irwin (1932) flocculation in his rabbits took place after 5 minutes.

The course of events seen in the liver will now be described.

Fig. 3, A shows the section of normal liver of frog; the characteristic cells containing melanin are shown at *m*. Fig. 3, B shows that by the time 24 hours have elapsed after injection thorotrast is found in the liver sinusoids. Some of this is clearly inside cells. After 48 hours cells containing thorotrast are found in practically all the sinusoids, and after 14 days even larger aggregations of these cells are seen. At 52 days (fig. 3, C) the cells in the sinusoids have become more numerous, but at 133 days (fig. 3, D), while there are still some aggregates of large cells in the sinusoids, thorotrast is clearly visible in the liver-cells themselves. Thorotrast is also absorbed by the cells containing melanin. (Tripoli (1934) using smaller doses found that in the mammals on which he worked, although there might be an occasional thorium particle in the liver-cells after 1 month, later no such particles were to be seen; all being within the reticulo-endothelial cells.)

Some of the details of this sequence of events is shown after high magnification in fig. 3, E (after 52 days) and F (after 133 days). After 48 hours the thorotrast is mainly confined to the sinusoids; the 52-day (E) specimen showed some tendency for the thorotrast to be accumulated in the liver-cells away from the sinusoids. At 133 days (F) many of the sinusoids were clear, but thorotrast was visible in the cells where in some cases it appeared to surround the nuclei.

We interpret these results as follows: in the liver thorotrast is first picked up by reticulo-endothelial cells and macrophages which appear to lie free in the sinusoids, but which may be reticulo-endothelial cells which first enlarge and then perhaps become free in the sinusoids; that these cells then tend to coalesce, or at least aggregate, and that then some process of transference of the thorotrast from these cells to the parenchyma cells takes place. This results in a diminution after some weeks in the number of cells containing thorotrast; these cells almost block the sinusoids. As will be seen later, this apparent sequence of events is not the same as that described for the rabbit by Irwin (1932).

FIG. 3 (plate).

Photomicrographs of sections of liver of (A) normal and (B–F) experimental frogs injected with 0·1 c.c. thorotrast. A to D magnified as fig. 5, C.

B, 24 hours after injection.
C, 52 days after injection.
D, 133 days after injection.
E, 52 days after injection } magnified as fig. 5, F.
F, 133 days after injection }
cs, cells, containing thorium particles, in sinusoids.
m, cells containing melanin.
t, thorium particles in liver-cells.

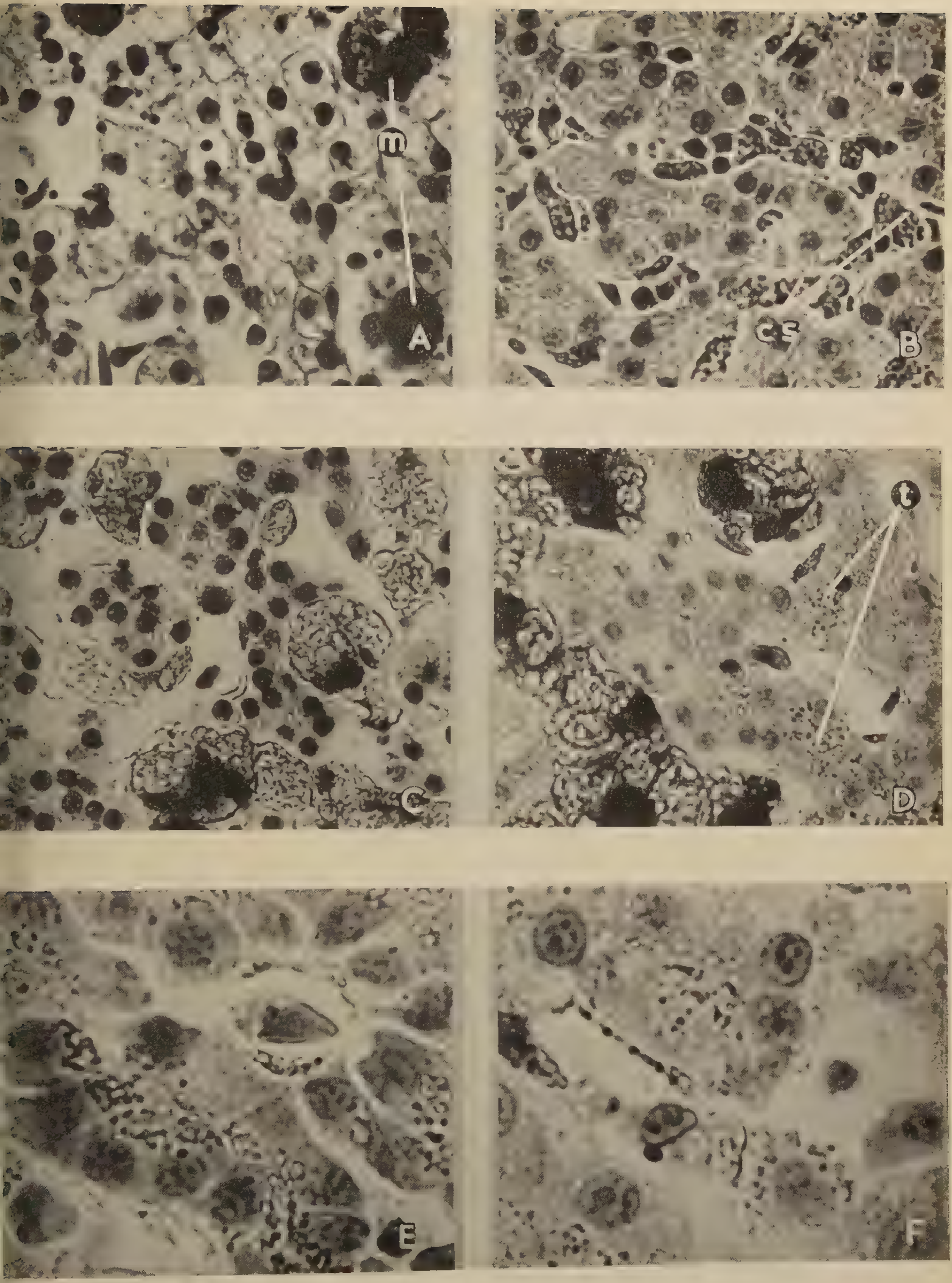

Fig. 3

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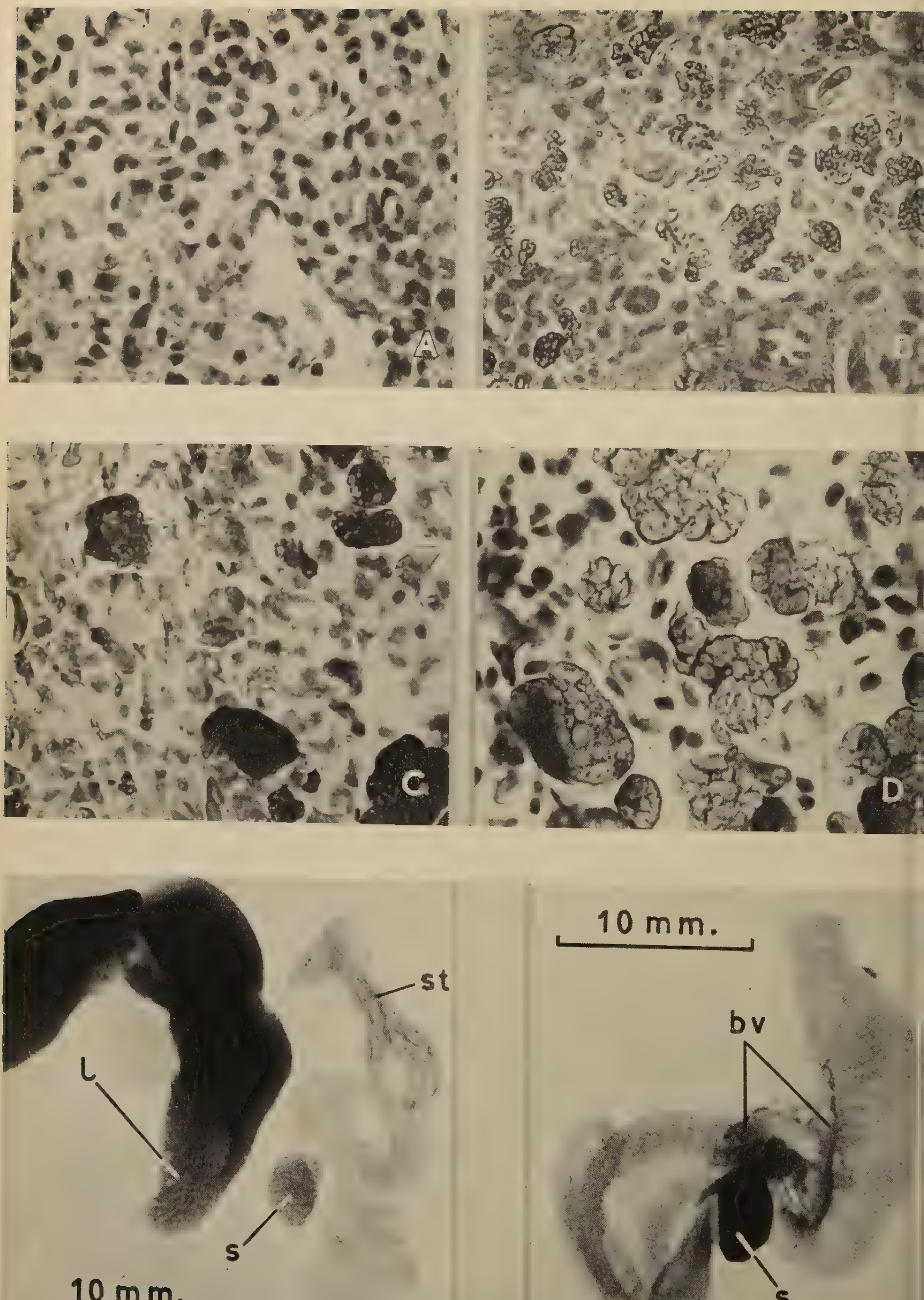

FIG. 4
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In the spleen the succession of events is shown in fig. 4, A–D. A shows normal frog spleen. B (after 72 hours) shows the accumulation of thorotrast in the spleen, some particles definitely in cells but some only doubtfully so. C (after 42 days) shows a tendency to form larger aggregations, and D (133 days after injection) shows some very large masses of thorotrast indeed. The coming together of phagocytic cells containing thorotrast to form composite structures in the spleen of the rabbit has been described by Irwin (1932). It will be remembered, however, that when a spleen was observed at intervals over a considerable period it showed a diminution in size (fig. 2, I–L).

The combination of these results with those obtained from radiography suggests that there might be a gradual transference of thorotrast from the spleen to the liver, and previous radiographs, as has already been remarked, suggest that there might be other movements going on, particularly in the early stages, as transient shadows had appeared and disappeared; this suggested that thorotrast might be present in other parts of the body shortly after injection, particularly in the walls of some parts of the alimentary canal.

Some heavier injections show the accumulation of thorotrast in the walls of the alimentary canal. For example, in fig. 4, E is shown the alimentary canal of a frog injected 20 days previously with 0·4 c.c. thorotrast. The accumulation in the liver and spleen is well shown, but there is also much thorotrast in the walls of the stomach. The lines running along the length of this organ were found, on sectioning, to be caused by the deposit of thorotrast in the ridges of the mucosa. The finding of thorotrast in the mucosa is unusual as it is usually confined to the submucosa. However, the injection was a heavy one, and the fact that the thorotrast is in the cells of the surface layer of the stomach suggests an explanation for the condition found in the first experiment described, where thorotrast was apparently found in the lumen of the alimentary canal.

With the injection of 0·1 c.c. thorotrast the shadows in the wall of the alimentary canal are much less distinct than this, but in order to find out whether it was possible to follow the passage of the thorotrast from the wall of the alimentary canal to the liver, a frog, into which 0·1 c.c. of thorotrast had been injected 30 hours previously, was killed and subsequently sections

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FIG. 4 (plate).

A–D, photomicrographs of sections of spleen from (A) normal and (B–D) experimental frogs injected with 0·1 c.c. thorotrast. Magnified as fig. 5, C.

A, normal spleen.

B, 72 hours after injection.

C, 42 days after injection.

D, 133 days after injection.

E, radiograph of alimentary canal, liver, and spleen excised from a frog injected with 0·4 c.c. thorotrast 20 days previously.

F, radiograph of stomach, intestine, and spleen excised from a frog injected with 0·5 c.c. thorotrast 9 days previously.

bv, blood-vessels.
l, liver.
s, spleen.
st, stomach.

showed that thorotrast was present (possibly in endothelial cells) in the glomeruli of the kidney (fig. 5, A), in the interstitial tissue of the pancreas (fig. 5, B) and most particularly in the submucosa of the duodenum (fig. 5, C), where i appeared to be clearly in macrophage cells. Fig. 5, D shows the wall and lumen of a vein in the duodenal region, and cells containing thorotrast are seen in the wall of the vessel. Sections of organs of animals similarly treated but which were cut at later times (even 14 days later) do not show the presence of such cells, and this suggests that the cells migrate with the contained thorotrast. It is a well-known fact that the venous drainage of the wall of the alimentary canal favours transport through the portal system to the liver (Wright, 1954, p. 56). With this in mind serial sections were cut through the region of the portal vein of a frog injected 30 hours previously, and in several sections obvious white blood-cells containing thorotrast were found free in the lumen of the vein, and one of these is shown in fig. 5, E and under higher power in fig. 5, F.

Fig. 4, F, which shows the alimentary canal of a frog killed 9 days after injection with 0·5 c.c. thorotrast, shows a marked accumulation in the spleen and also in the surrounding blood-vessels, again suggesting that the thorotrast has been cleared from the wall of the alimentary canal and is being transported through the portal system.

Two other regions which have not so far been mentioned have been examined for the presence of thorotrast.

The first situation is the marrow of the long bones. The presence of thorotrast here is not suggested by the X-rays, but nevertheless smear preparations of marrow from the femur of a frog killed 133 days after injection showed the presence of a considerable quantity included in marrow-cells.

The second situation is the vessels of the lungs. Irwin (1932), working on the rabbit, found cells containing thorotrast in lung tissue 3 months after injection although previously they had not been present. He suggested that cells containing thorotrast wandered out from the liver through the circulation to the lungs and that they passed out into the alveoli and thus the thorotrast was excreted from the body. Such excretion cannot assume large quantities in mammals. In man thorotrast persists in the liver and spleen for considerable periods, presumably for life (see Cassel and others, 1951). We found no such cells in the lungs even in the frog which survived for 133 days.

FIG. 5 (plate).

A–D, photomicrographs of sections of organs of a frog injected 30 hours previously with 0·1 c.c. thorotrast. A and B to same scale as C.

A, kidney showing thorium particles in the glomerulus, *tg*.

B, pancreas showing thorium particles in interstitial cells, *ti*.

C, duodenum showing cells in submucosa containing thorium, *tsm*.

D, blood-vessel of portal system showing *l*, lumen; *w*, wall, and *tw*, thorium particles in cells in the wall.

E–F, a cell containing thorium particles in a blood-vessel.

E, a cell *c*, lying in the lumen of a blood-vessel together with red blood corpuscles, *rbc*. The frog had been injected 30 hours earlier with 0·1 c.c. thorotrast.

F, the same cell as shown in E but more highly magnified, showing the thorium granules, *tg*.

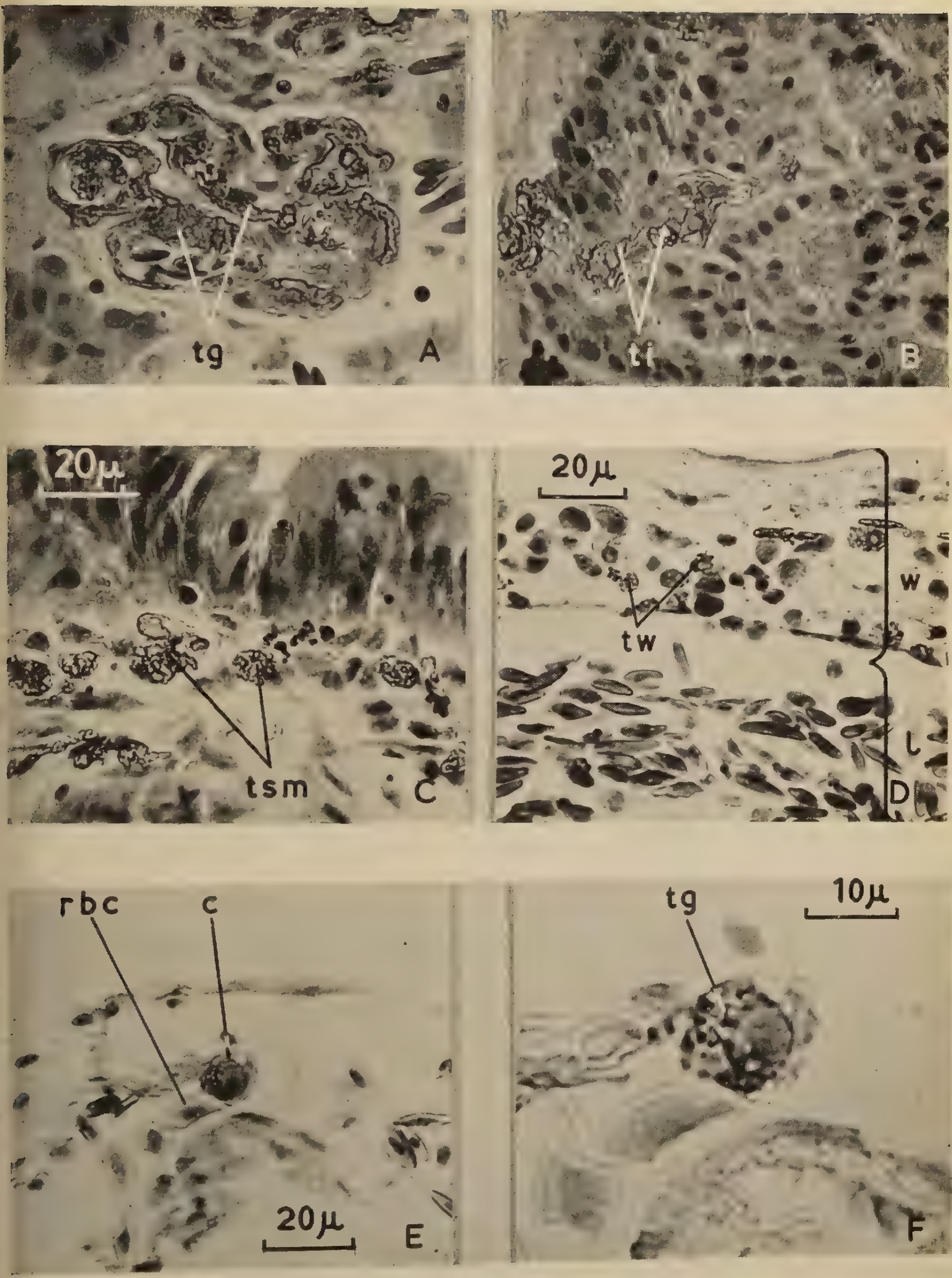

FIG. 5

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Discussion

Our results lead us to the following conclusions: thorotrast injected into the dorsal lymph sac of the frog passes rapidly through the anterior lymph hearts into the blood-stream. From the blood-stream it is progressively removed by the action of cells of the body possessing the properties of macrophages.

These may include certain of the reticulo-endothelial cells of liver and cells of the bone-marrow and also similar cells in other parts of the body, particularly those occurring in the walls of the alimentary canal. These cells are to be regarded as 'resting-wandering' cells or macrophages, and we consider that the results both of our radiographic studies and of the micro-anatomy support this hypothesis.

If this hypothesis is correct, then the rapidity of the removal of the thorotrast from the blood-stream will depend on the number of macrophages which are initially available for removing the thorotrast.

In the first experiment recorded, when 0·5 c.c. of thorotrast was injected, it was visible circulating in the blood up to and including the 2nd day after injection, but it had all disappeared by the 5th day. When 0·1 c.c. was injected, the shadow in the blood-vessels of the legs which was visible after 3 hours 45 minutes had disappeared after 24 hours. This suggests that, when the quantity injected was great, additional macrophage cells were produced to remove the thorotrast and that this took time. Maximow and Bloom (1948, p. 98) state that macrophages in defensive action cannot be sharply separated from lymphoidal cells and that it seems probable that in all defence reactions some new macrophages arise locally from the mitotic division of pre-existing macrophages or by the direct assumption of phagocytic activity by cells having mesenchymal potencies. These authors also point out that, when required to combat general infections or to take up vital dyes, large numbers of free macrophages are mobilized in the liver, bone-marrow, or spleen. Some which pass into venous sinuses are carried by the blood-stream into the right side of the heart and so eventually to the lungs, where most are filtered off in the capillaries, few passing into the general circulation.

Our results are not, in general, out of keeping with this general statement, but it would appear that in the frog the macrophages in the gut wall which contain thorotrast generally migrate into the portal system and that they are transported to the liver where they come to block the sinusoids; here they may be joined by similar cells coming from the spleen. Eventually, we believe, their contents pass into the liver-cells themselves and the macrophages disappear, but as to what is their actual fate we have no evidence.

Irwin (1932), working on the rabbit, interpreted his observations differently. He concluded that the thorotrast was removed from the blood into the parenchyma and Kupffer cells of the liver almost immediately after injection. Later the quantity visible in the parenchyma cells declined while that seen in the Kupffer cells increased; eventually, starting after an interval of 3 months, the Kupffer cells became free in the blood and were passed to the lungs where they lodged in the fine capillaries and the cells passed through into the alveoli Here they died and their remains were removed by ciliary action of th bronchi. Hence there was really an excretion of thorotrast. As alread remarked, in a frog 133 days after injection no trace of thorotrast could b found in the lungs. Tripoli (1934) also found that in various mammals thoro trast was eliminated from the parenchyma cells of the liver within a compara tively short time (1 month).

A point of interest is the obviously large amount of thorotrast which i removed from the blood by macrophages in the wall of the alimentary canal This we believe may be correlated with the zoological position of the frog Jordan (1933) has reviewed the evolution of the spleen and shown that the spleen tissue is embedded in the wall of the intestine in certain fishes. Here it combines erythro- and lymphocytopoietic functions. In higher animals these functions become separated, the marrow of the long bones taking on the former and the spleen the latter. In the Anura, of which the frog is one, the spleen has taken on the lymphocytopoietic function but also forms erythrocytes at certain stages of the life cycle. The group is evolutionarily the lowest in which the bone-marrow has an erythrocytopoietic function. It is known, however, that the lymphocytopoietic function is still retained in part by the alimentary canal in mammals and probably even to a greater degree in frogs, and much of the division of the macrophages appears to take place in this region. It is also true that the macrophages must undertake the ingestion of the thorotrast particles in sluggish parts of the circulation, and it would seem that the capillaries of the wall of the alimentary canal provide a favourable location for this. Also, particles of thorotrast which have passed out of the blood-stream altogether in the connective tissue fluid are engulfed by macrophages in the connective tissue. That thorotrast can occur in cells other than macrophages and parenchyma cells of the liver has already been shown in connexion with the stomach (fig. 4, E). Indeed it is well known that many cells, besides those of the reticulo-endothelial system, take up colloidal particles if a sufficiently large quantity is injected.

The variation that was found in the appearance of liver and spleen in various frogs some 28–30 hours after injection has been noted. This is of particular interest in view of the work of Gaunt and Wright (1940) on rabbits, who found a very constant relationship between the amounts in the three regions, liver, spleen, and bone-marrow. They found about half the thorium could be detected chemically in the liver, one-quarter in the bone-marrow, and one-eighth in the spleen. We have not made any chemical analyses, but it would seem unlikely that such a constant relationship could have been detected. Again, this may be correlated with the lower evolutionary position of the frog, the mechanism responsible for the removal of the thorotrast not having reached such a stable condition as is found in mammals. As already noted, however, it may be correlated with the nutritional condition of the animal at the beginning of the experiment.

In conclusion, we should remark that while the details of our observations at some points agree with, and at others diverge from those of workers who have studied the reactions of mammalian tissues to the injection of thorotrast, the two points which we consider of most interest in the present study are, first, the actual radiographic demonstration of the movement of the lymph in the lymph spaces of the frog; and second, the evidence which appears to show that cells with phagocytic properties and which engulf thorium dioxide particles migrate about the body, and that these migrations lead ultimately to the liver.

We wish to express our thanks to the Royal Society (Scientific Publications Grant-in-Aid) for a grant towards the cost of reproducing the plates which illustrate this paper.

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The Nervous System of the Ephyra Larva of *Aurellia aurita*

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SUMMARY

The rapid co-ordination of the beat had been identified with a specialized system of neurones which spreads over the muscles from the marginal ganglia. By analogy with other invertebrates this has been called the giant fibre system.

The feeding response and the spasm are co-ordinated by a separate net, called 'the diffuse nerve-net', which is both physiologically and histologically distinct from the giant fibre system.

The histological structures of the two nerve-nets and the marginal ganglia are described.

Although the diffuse nerve-net contains sensory cells, it also acts upon the muscles, and a double motor innervation is indicated.

The two nerve-nets meet and interact at the marginal ganglia. An attempt has been made to interpret the neurone structure of the ganglia.

THE co-ordination of the rhythmical swimming movement of the adult *Aurellia* is brought about by a network of relatively large bipolar nerve-cells with histological and physiological characters closely parallel to the through-conducting system in the mesenteries of some actinians. The relatively rapid conduction of impulses depends on the large size of the neurones and brings about a widespread contraction which has a mechanically desirable symmetry. By analogy with functionally similar systems in other invertebrate groups, it is proposed to call these nerves the 'giant fibres' to distinguish them from other nerves which are known histologically and physiologically in the nervous systems of coelenterates. In the adult these giant fibres are 6–12 μ in diameter. This is small relative to the giant axons of crustacea, annelids, and molluscs, but is large relative to the other fibres of the nervous system of *Aurellia*. In the Ephyra larva the whole structure is on a smaller scale. The animal is 0·3–0·5 cm in diameter compared with 15–25 cm for the adult, and the giant fibres are usually less than 1 μ thick, though stouter and longer than the other nerve-cells. The use of the expression 'giant fibres' for this part of the nervous system is therefore based on the functional significance. Here is a system of nerve-fibres which are relatively large, in close association with the muscle, and a single stimulus produces a simultaneous contraction of the whole bell.

In earlier observations on the nervous system of the adult *Aurellia* (Horridge, 1954*b*) experimental work was confined to the giant fibre system. But Romanes (1877) had demonstrated a wave of tentacle retraction travelling round the margin with a velocity of 0·23 m/sec, which he distinguished from the contraction wave in the circular muscle of the bell with its velocity of 0·46 m/sec. The interesting point is that the tentacle wave could initiate a

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contraction wave when it reached a tentaculocyst. This has been briefly confirmed, but whereas the giant fibres propagate the contraction wave there ar insufficient histological data for *Aurellia* in which to find an anatomical basi for the tentacular waves. Bozler's work on *Cotylorhiza* (1926*a*) and *Pelagi* (1926*b*) contains examples of propagated excitation distinct from the gian fibre system and the conclusion is there reached that *three* overlying conducting systems are present, at least for parts of *Cotylorhiza*. In his histologica work on *Rhizostoma* (1927) Bozler divides the large bipolar cells into thre types, according to size, but hesitates to say that his preparations show a anatomical separation into distinct overlying networks. Because medusa show a great diversity of the responses, the histological and physiologica

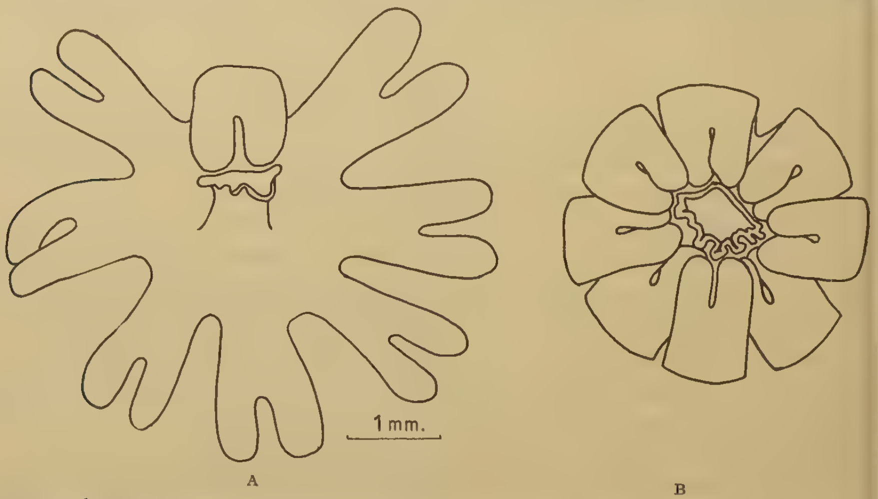

FIG. 1. A, the co-ordination of the mouth and one arm in the feeding response. B, the long-lasting contraction of all the arms in the spasm.

data to substantiate each other must come from the same animal. It is fortunate that the ephyra larva of *Aurellia* provides two distinct kinds of response and that the corresponding elements in the nervous system can be described from methylene blue preparations.

In his study of the ephyra larva of *Aurellia* Gemmill (1921) gives a brief account of the method of feeding. On touching a particle of food in the surrounding water, an arm rapidly bends towards the mouth, which at the same time turns out to meet it (fig. 1, A). The contraction is sustained and each arm acts independently, so that several copepods, for example, may be caught in rapid succession by different arms. This is referred to as the 'feeding reaction'. Stronger stimulation is followed by a sustained contraction of the whole animal, here referred to as the 'spasm', following Romanes's terminology. The arms fold tightly over the oral surface in a contraction that may last several minutes (fig. 1, B), and the animal falls through the water. Quite distinct is the normal rhythmical and symmetrical swimming movement which is here referred to as the 'beat'. The larvae maintain themselves near the surface by swimming actively in the symmetrical manner typical of most medusae.

HISTOLOGICAL RESULTS

Methods

The most successful preparations were obtained with a solution of reduced methylene blue made up as follows. To 10 c.c. of 1% methylene blue (I.C.I. 'zinc free') in distilled water are added two drops of 10% hydrochloric acid. The mixture is warmed in a test-tube in a beaker of boiling water and a 12% solution of rongalite white (Gurr) is added drop by drop until the colour is just discharged. The solution is allowed to cool and used the next day, when the precipitate that has formed is removed with a long pipette. This is essentially the method of Unna (1916). The tube was kept corked in this case, though according to Dr. Alexandrowicz (private communication) this is a disadvantage.

For an appraisal of a study based on intra-vitam methylene blue staining, one must be aware of the limitations and advantages of this method. Often a preparation showed only the processes of a few cells, which could then be individually followed. Some preparations showed only cells of one type, while other preparations showed a different group of cells and fibres. This was a valuable aid in the separation of the several types of cells to be described, and the total plan was obtained by combination of results. But one limitation of this method is the scarcity of preparations which show the exact relation between different systems which stain at different times. For this reason the conclusion that a double innervation of the muscles occurs is based on physiological evidence and is not as yet confirmed by histological observation. Similarly, no direct evidence has been found of histological connexions between the two systems. Even if these were observed, physiological experiments would be required to give them a significance in the life of the animal.

The giant fibre system

Overlying the radial and circular muscle is a network of highly orientated bipolar nerve-cells. As shown at *a* in fig. 2, the area covered by these fibres is sharply limited to the sheets of muscle-fibres except where the nerves connect with the marginal ganglia and where they make a short cut across the corners at the base of the arms. The cell-bodies (fig. 3, A) are similar to those of the giant fibre system of the adult (Horridge, 1954*a*), but they are smaller, being 6–10 μ long. The smaller cells, however, do not have the characteristic fusiform shape which may be due to passive stretch as the animal grows. The large granules in the cytoplasm stain deeply and are similar to those of the adult. The axons are usually less than 1 μ thick, but parts of the axons, particularly near the cells, could be seen to have a flattened structure. The beads produced by overstaining tend to be elongated. Synapses (fig. 3, B) were frequently observed, and were similar to those of the giant fibre systems of *Rhizostoma* (Bozler, 1927) and *Metridium* (Pantin, 1935). Most of the synapses were of the single contact type, but there were plenty of synapses with multip contacts, where fibres ran alongside each other for as much as 30μ. Specialize

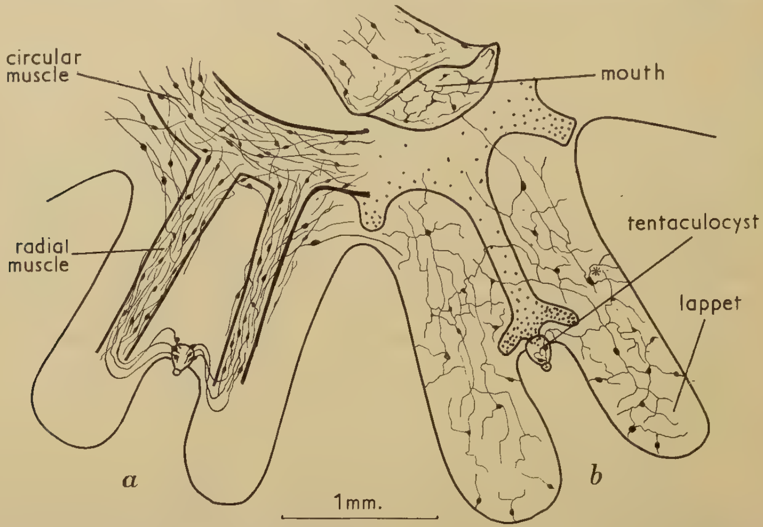

FIG. 2. Two arms of the bell showing the main structures described. *a*, the muscle-strips an the giant fibre system. *b*, cells of the diffuse nerve-net and the underlying gastric cavit

nerve-endings on the muscle were searched for in vain, but a sharp twist of a axon round a muscle-fibre was often seen (fig. 3, C).

Between the arms the giant fibre system extends a little way on the side o

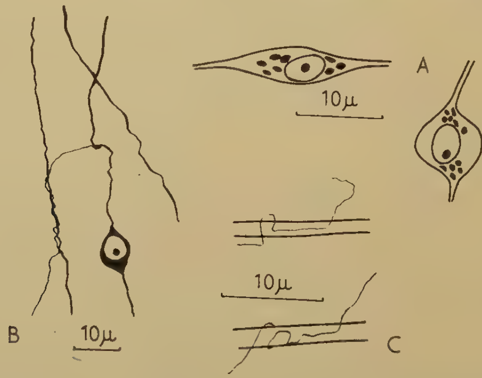

FIG. 3. The giant fibre net. A, cell-bodies. B, three synapses. C, relations with muscle-fibre

the disk, whereas elsewhere it is strictly limited to the epithelium overlyin the muscle-fibres. This may have a functional significance as a short cut. Th numbers of fibres cannot be safely estimated from methylene blue preparations; at most, 25 nerve-cells have been counted overlying a single strip o radial muscle (area approx. 0·3 sq. mm.) and the diagram of the giant fibr system (*a* in fig. 2) shows the approximate number of cells in the mos favourable preparations. The nerve-cells are quite distinct from the muscle-cells, which also stain with methylene blue, particularly at the peripheral ends of the radial muscles (fig. 4).

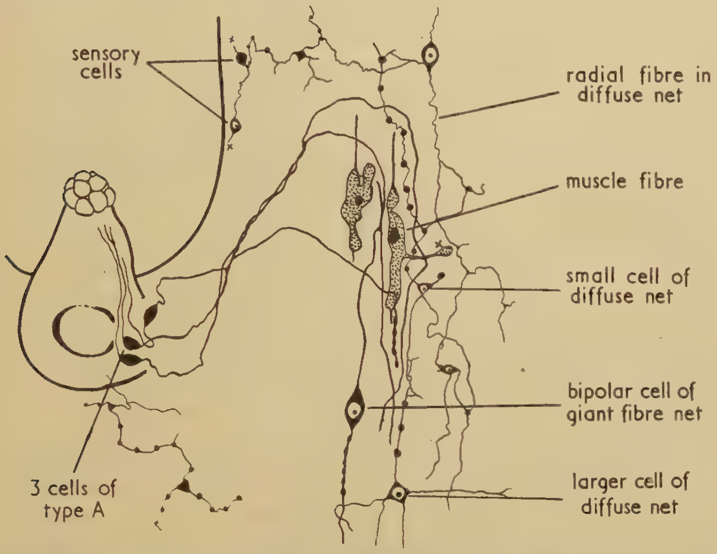
sensory cells
radial fibre in diffuse net
muscle fibre
small cell of diffuse net
bipolar cell of giant fibre net
larger cell of diffuse net
3 cells of type A

FIG. 4. The connexion of the giant fibres with a ganglion, showing also cells of the diffuse net at the peripheral end of a radial muscle-strip.

The diffuse nerve-net

Besides the giant fibre system, other nerve-cells and their axons form a nerve-net which spreads over the whole of the epithelium. This nerve-net, which is here called 'diffuse' (in the sense of *dispersed*), is composed of elements of more than one kind. There are bipolar cells, apparently sensory, with a short process that comes to the surface of the epithelium; there are bipolar and multipolar cells which form a net over the whole of the oral and aboral surfaces; and there are bipolar cells with two long axons orientated along the arms and round the mouth. Physiological evidence shows that some of these axons undoubtedly act on the muscles, though histologically definite endings on muscle-fibres have not been found, but this diffuse net is best seen in the muscle-free areas, on the aboral side, and along the margins of the lappets.

A net of bipolar and multipolar cells lies in the epithelium over all the aboral surface. Most of the cells are multipolar and in many a short process from the cell ends at the surface of the epithelium. There is a tendency for the axons to be radially arranged in the zone round the centre (fig. 5, A), probably an effect of the growth of the disk. This network of nerves extends round the sides of the disk and arms to the oral surface, and from it two fibres run between the lappets, one to each side of each marginal ganglion.

The diffuse net has several characteristics which distinguish it from the giant fibre net. On the aboral surface the fibres apparently all connect with

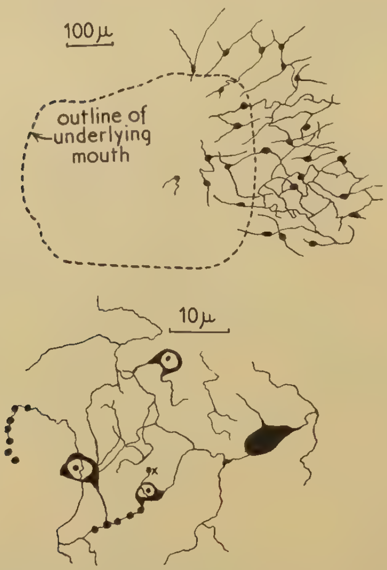

Fig. 5. The diffuse net on the aboral surface. A, small scale; B, large scale.

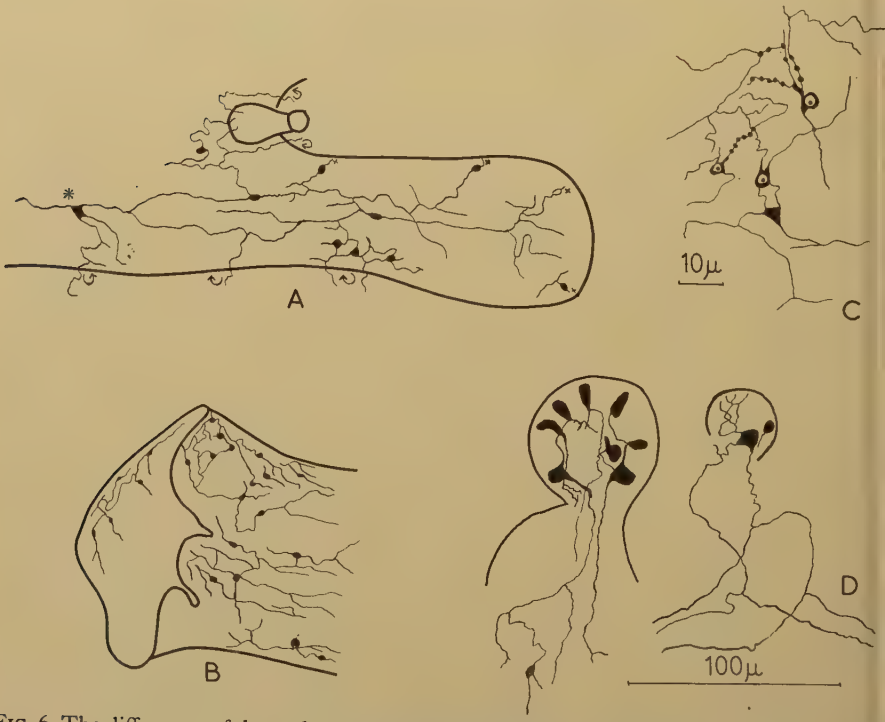

Fig. 6. The diffuse net of the oral surface. A, lappet of arm. B, side of mouth. C, on mouth fold
D, developing tentacles.

each other or with nerve-cells. Even in these living preparations studied under oil-immersion it was impossible to decide any question of continuity or contiguity on account of the small size of the cells and fibres. The fibres are at the limit of resolution, where a line can be seen but no structure made out at its ending. The axons are extremely thin, with many twists and sharp angles, in contrast with the broader, straighter giant fibres. The beads along them in methylene blue preparations appear very readily and are spherical. The cells are usually multipolar, or apparently so, but of those that are bipolar some are typical sensory cells with one short process to the surface of the epithelium. The cell-bodies are never fusiform and are almost entirely filled with a relatively large nucleus (figs. 5, B and 6, C). The contrast between the two nerve-nets is seen in fig. 4, and by comparison of fig. 6 with fig. 3.

The diffuse net occurs on the aboral surface and extends round the sides of the arms and the disk and spreads over the whole of the oral surface. But here its structure is complicated by the connexions with the marginal ganglia and by the presence of bipolar cells with long processes which are arranged radially along the arms and up the sides of the mouth, with a few long processes extending round the mouth (*b* in fig. 2; figs. 4 and 6, A). On one arm as many as six of these long cells have been seen, some stretching almost the whole length from the lappet to the disk. They make connexions with the peripheral bipolar cells of the sensory type and with the multipolar cells of the remainder of the diffuse net. A constant feature was a nerve-cell about the middle of the outside edge of the radial muscle, with a centrally directed radial axon and a number of branches which spread round the side of the arm to other cells of the diffuse net on the aboral side (marked with a star in *b*, fig. 2, and in fig. 6, A). The central connexions, when followed, have been lost among the abundant fibres of both nerve-nets over the circular muscle, but the physiological work shows that there must be connexions with the nerves round the mouth. The processes of the multipolar cells appear to form a network over all the epithelium of the oral surface, but most preparations were patchy and this conclusion is reached by combination of many observations.

Long processes of other bipolar cells run up the sides of the manubrium and connect with a network of nerve-fibres which spreads over the folds of the mouth and extends down the inside towards the stomach cavity (fig. 6, B and C). Other relatively long fibres run round the mouth with connexions to this network and to very fine fibres from multipolar cells that were often seen in the epithelium directly overlying the developing gastric filaments below.

In some specimens the first rudiments of the marginal tentacles had appeared as round buds on the oral surface between the arms. Here nerve-cells appear which are characteristic of the diffuse nerve-net and which make connexions with fibres of the diffuse net round the mouth. The earliest tentacle rudiments show one or two bipolar cells (fig. 6, D), each with one long axon directed towards the mouth, and the other process branching profusely within the bud. In one case several epithelial sensory cells stained, with short axons that could not be traced out of the bud.

The marginal ganglia

Each tentaculocyst is a conical papilla about 0·2 mm high and 0·1 mm broad at the base, with a round conglomerate of crystals at the apex. It i hollow and the cavity connects with the coelenteron. Although the nerve-cell stain readily with methylene blue one cannot conclude that all types have been seen. The relationships of the cells and fibres show a marked bilateral symmetry and the histological appearance is reminiscent of the ganglia of othe invertebrates in which most of the connexions between nerve-cells are presumed to occur in a neuropile; see, for example, fig. 7, A and C. Four kinds o cells have been distinguished; to avoid assumptions of function these hav been labelled *a*, *b*, *c*, and *d*.

a. A group of bipolar cells is found at each side of the base of the tentaculocyst. Each has a long process, of the giant fibre type, which sweeps round to the end of the radial muscle and connects by contact synapses to the gian fibre system. The other process of each cell ascends into the ganglion and branches to fine ramifications among the cells and fibres of type *b*.

b. The apex of the ganglion contains bipolar and multipolar cells with short processes that branch many times to form a dense network of fibres These ramify among the crystals at the apex but never descend out of the ganglion.

c. Near the base of the ganglion many preparations showed a pair of bipola cells, one on each side. From each of these a short process branches among the fibres and cells of type *a* and a long axon reaches across the aboral face to do the same among the corresponding cells at the other side.

d. In the side walls about half-way towards the apex are found bipolar cells, each with a short process that ascends and branches among cells and fibres of type *b*, and a long axon of the diffuse net type that extends out of the ganglion and connects with fibres of the diffuse nerve-net on the oral surface.

The appearance of the nerves in the ganglion is best understood by reference to the drawings (fig. 7). No fibres have been found on the aboral side of the base of the tentaculocyst; most of the ascending and descending axons are in the side walls and appear to lie under the ectoderm. The ectodermal origin of the cells of types *b*, *c*, and *d* is supposed from the orientation of the cell and its position in the epithelium and the occasional observation of a process to the surface. Endodermal nerve-cells have not been identified on the ganglion, but the origin of cells of type *a* is unknown. One point is obscure in the diagrams: at the places where two fibres appear to cross when projected to the plane of the paper, they are usually in fact quite separate, but this could only be shown by a convention. The tendency was to look for connexions between fibres, which were often emphasized by a large bead of methylene blue at the crossing, but there is no evidence that this indicates a functional pathway. These junctions have been indicated by drawing a dot at the crossing, as in the electrical convention. Some cells have a connexion with the surface, and the end of this is drawn with a small *x*.

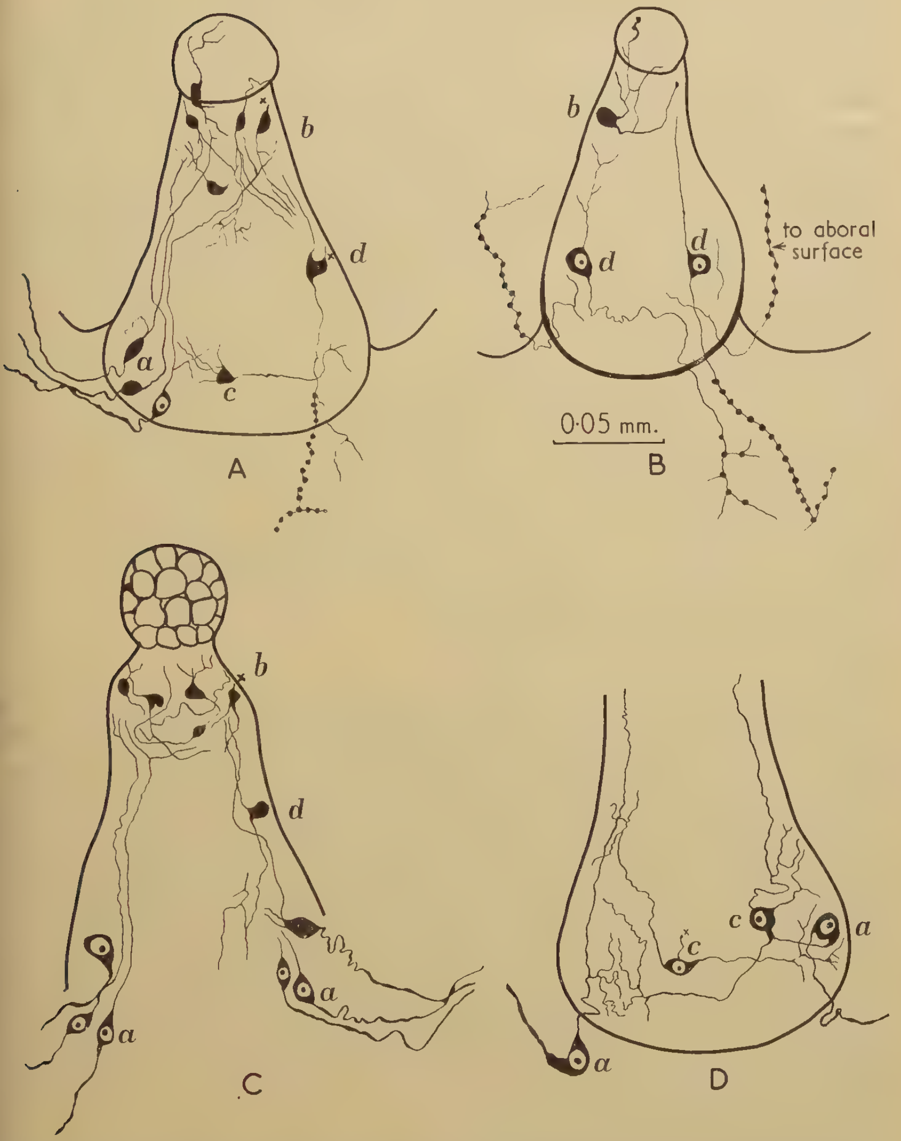

Fig. 7. Examples to show the neurone structure of a ganglion. A, the four types of cells, *a–d*. B, the connexions with the diffuse nerve-net. C, the connexions with the giant fibre net. D, neurones of type *c* make a connexion between the cells of type *a* on the two sides.

Physiological Results

The action of the muscles

Two bands of radially orientated muscle-fibres lie along each of the arms (fig. 2). These extend from the lappets to the circular muscle, which forms a continuous band near the margin of the disk.

A few simple experiments were sufficient to show the part played by thes muscles in the normal activities. When all the arms but one were removed th disk and the remaining arm continued to beat in the usual way, and it coul then be readily observed that radial and circular muscles contract together a each beat. But a touch on one of the lappets elicited a feeding response fro the arm and a co-ordinated movement of the mouth, with no contraction o the circular muscle. A delicate mechanical stimulus to the circular muscle however, sometimes produced a local, maintained contraction; whilst strong stimulation by touching with a seeker any part of the remaining arm and disk was followed by a spasm in which both radial and circular muscles contracte for as long as 30 seconds.

These results were consistent with all observations on whole animals o isolated parts. For example, an isolated arm continued to beat with radia muscle alone. In isolated arms one of the two strips of radial muscle would sometimes beat independently of the other, an observation which is important for the interpretation of the structure of the ganglion in relation to its function. As in the normal animal, a touch on one of the lappets of an isolated arm may produce a feeding response of that lappet alone, or both strips of the radial muscle may make the slow contraction, apparently with the same muscle that makes the quick contraction of the beat.

Electrical stimulation

The effect of electrical pulses of minimum strength from a neon stimulator was examined by using as an electrode a capillary tube drawn out to a fine point. Single shocks produced a single twitch of both radial and circular muscle in whole animals or isolated parts. This response was often followed by a spasm which is considered to be due only indirectly to the stimulus. A second pulse of the same strength following within 2 seconds was always followed by a spasm. There was no indication of a long refractory period in either group of muscles. It was often possible to elicit a feeding response by running the stimulator at ten pulses a second and slowly increasing the strength of the stimulus until the co-ordinated movement appeared. This had the same effect as a touch with a particle of food, but the danger of artifacts arising from direct spread of the stimulus is too great for the results to be used in the further analysis.

The separation of the two nerve-nets

The argument for the existence of two distinct nerve-nets depends upon the observations that the feeding response and the beat can each occur alone. The feeding response was observed many times with no accompanying beat, and the bell regularly beats with no sign of a maintained contraction. This applies both to whole animals and to isolated parts, which spontaneously beat if they contain a tentaculocyst. Another observation important for the argument is of long-lasting but local contractions of parts of the circular and radial muscles in response to mechanical stimulation with a bristle. These maintained contractions may be propagated a short distance, or over the whole disk exactly as in the spasm.

Many observations were made of the effects of cuts in different directions, but most of these only agree with the interpretation given here without proving conclusively the relation between the histological structures and the conduction of excitation. But four experiments, when considered with the anatomical plan, appear to be decisive. These depend upon the contrast between the localized distribution of the giant fibres and the general distribution of the diffuse nerve-net.

1. A short incision is made from the base of each arm towards the mouth (fig. 8, A), sufficient to cut through the band of circular muscle and its overlying giant fibre nerve-net. Each arm now beats at its own rate, independently of the others. However, in a spasm the whole animal co-ordinates normally.

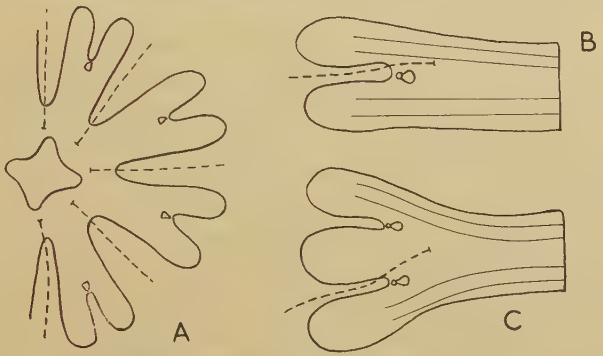

FIG. 8. Arrangement of experimental cuts. A, radially between the arms. B, at the side of a ganglion on an isolated arm. C, between two ganglia on an abnormal arm.

2. An arm is removed and it continues to beat. A cut is now made at the side of the tentaculocyst (fig. 8, B), where the nerve-fibres from the ganglion sweep round towards the end of the radial muscle. Only the strip of muscle still in communication with the ganglion continues to beat, but the feeding response or the spasm, when evoked, may involve the whole of the arm.

3. In one abnormal case two tentaculocysts were found on the same arm (fig. 8, C). After isolating the arm, a cut between these produced a preparation in which the two sides of the arm beat independently, but the co-ordination of the spasm remained intact.

4. The terminal ephyra larvae, still attached to the scyphistoma, beat independently of each other, but a touch with a seeker at the base of a scyphistoma initiates a wave of excitation which travels along the animal, made visible by the successive spasm of each developing ephyra.

Discussion

The two nerve-nets

We are presented with a short contraction of the whole bell at each beat and an independent lasting contraction of part only in the feeding response. Using the known properties of nerves and nerve-nets, it is impossible to explain this co-ordination as the action of one conducting system, e.g. by having a single impulse for the beat and several impulses for a feeding response. The same state of affairs has already been encountered in the adult *Aurellia* (Horridge, 1954*b*), in which there must be a second conducting system for the propagation of excitation other than the contraction wave of the beat. For the ephyra, two nerve-nets have been histologically demonstrated. The obvious conclusion is to identify the giant fibres with the co-ordination of the beat and the diffuse nerve-net with the feeding response and spasm. The experiments with various cuts in the bell were designed to test this conclusion and no evidence has been found against it. Also there is no suggestion that the two nets interact where they overlie the muscle-fibres.

The most satisfying aspect of this study is the agreement of the anatomical arrangement of the two nerve-nets with the physiological observations. The giant fibres rapidly propagate the contraction wave over both radial and circular muscle. Their distribution is restricted to the bands of these muscles except where they make a short cut between the arms. The marginal ganglia give rise to the spontaneous rhythm, and the connexions from them to the giant fibres (figs. 2, 4, and 7) no doubt conduct the excitation at each beat. By analogy with the adult, this is a single nerve-impulse.

The diffuse nerve-net is more widespread than the giant fibre net and in the experiments the co-ordination of the spasm survived the cutting experiments which destroyed the co-ordination of the beat. Similarly, working with the adult, Romanes (1877) found that 'excitational continuity' was more persistent than 'contractional continuity'. The diffuse nerve-net can be compared with the nerve-net of the polyp and with the similar nets of nerve-cells found in all groups of coelenterates. Common characteristics of all are the close connexion with sensory cells, the sensitivity to mechanical stimulation, and the co-ordination of feeding responses.

In the ephyra specialized cells have their place in the diffuse nerve-net. The bipolar cells with long axons orientated along the arms, the sensory cells of the lappets, and the fibres leading up to the mouth are clearly subservient to the feeding response. The connexions of the nerves of the larval tentacles with those of the diffuse net lead to the conclusion that the tentacular waves of the adult are propagated by the persistent diffuse nerve-net long after the macrophagous feeding response of the ephyra has disappeared. The feeding response and the spasm of the ephyra are both attributed to the diffuse net, which must therefore be considered physiologically as built in sectors, corresponding to the arms. A spasm is then a feeding response which, by interneural facilitation, has spread to the circular muscle and to all sectors.

The demonstration of two nerve-nets, which act in different ways on both radial and circular muscle, is very close to the acceptance of a double motor innervation. This conclusion has been derived from physiological evidence, and has not been confirmed histologically, partly because of the small size and partly for the reasons outlined in the section on 'methods'. The alternative to the double innervation is the hypothesis of two kinds of muscle-fibre, one for each nerve-net; observations on the course of contraction have revealed no evidence for this suggestion, and it seems unlikely.

The marginal ganglia

A partial interpretation of the structure of the ganglion can be based on the connexions with the two nerve-nets (fig. 9). First, the group of bipolar cells (*a*) at each side of the base of the ganglion are anatomically part of the giant fibre system and must transmit the impulse at each beat, even if they do not initiate it. Secondly, the cells (*c*) with processes between the two sides of the base of the ganglion presumably ensure that the impulse at each beat is propagated in both directions round the bell. The beat of one side only in an isolated arm is then due to the failure of this connexion. Thirdly, in the group of cells (*d*), each has an axon which joins the diffuse nerve-net and it has been observed that excitation in the diffuse net can inhibit, and perhaps also accelerate, the rhythm of a marginal ganglion. It seems likely that we have here a part of the pathway of this interaction. Finally, the connexions of the remaining group of cells (*b*) at the apex of the ganglion do not clearly indicate a definite function. The appearance of the histological detail here is reminiscent of that in ganglia of higher invertebrates, where the neuropile plays so large a part. However, processes from cells of types *a* and *d* both terminate among the apical mass of fibres and the whole arrangement can be interpreted as in the diagram (fig. 9). For example, a motionless ephyra larva may be stimulated to swim by a touch; but the beat is inhibited during a spasm. This observation can be interpreted in terms of the neurone picture set out above, but it also serves to show that the activity of the marginal ganglia is still little understood.

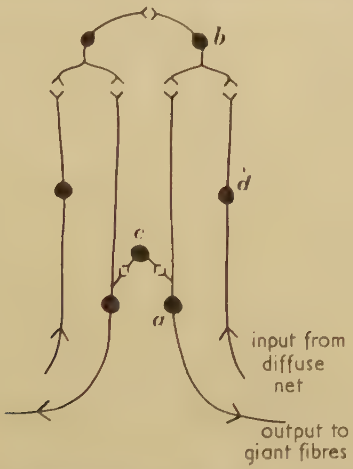

FIG. 9. Suggested functional arrangement of neurones in the ganglion with input from the diffuse nerve-net and output to the giant fibre net. This is hypothetical and derived from physiological results; its proper relation to the structure will be seen by comparison with fig. 7.

In such a system there is an abundance of alternative arrangements, but at least the scheme provides a basis for experimental work.

CONCLUSION

Integration of three kinds of evidence has formed a picture of the two separate nerve-nets. First there is the knowledge of the normal action of nerves; secondly, the physiological evidence indicates two separate conducting systems; and, thirdly, the histological study provides the anatomical background. The specialized giant fibre system propagates the contraction wave rapidly to all parts of the bell and is responsible for the symmetry of the beat. This is the nerve-net which Shäfer (1878) described in the adult and for a long time it was regarded as the whole of the nervous system outside the tentaculocysts. In all Scyphozoa studied it is typically a well-developed through-conducting net of non-polarized, large bipolar cells with contact synapses, similar to that in the mesenteries of some actinians. It is a specialized conducting system evolved in conjunction with the use of the bell as a swimming organ, and most of the work on the physiology of the nervous system of Scyphozoa has been concerned only with this giant fibre system. The comparatively straightforward, readily observed properties led to an underestimate of the complexity of the whole nervous system, but there has been great difficulty in explaining all the behaviour as the activity of this one nerve-net. Bozler's observations of many different reactions of Scyphozoa convinced him of the inadequacy of the theory of a single net, but he was never able to demonstrate his alternative of several overlying nerve-nets by combined histological and physiological evidence. After this, Pantin's work (1935 onwards) brought back the emphasis to one net, in which the importance of facilitation and the frequency of the excitation were stressed, and this net differed in physiological properties in different regions of the animal. In *Aurellia*, facilitation of contraction is also a well-marked feature of the easily recorded contraction wave (Romanes, 1877; Bullock, 1943) and decremental conduction (attributed to interneural facilitation) can be observed in the responses of both marginal and oral tentacles. But even with these possibilities the co-ordination of all the responses still cannot be explained as the work of one conducting system. However, the work on facilitation makes it necessary to devise discriminating criteria for the experimental demonstration of two nerve-nets. Several lines of evidence have been followed:

1. It has been shown in the adult *Aurellia* (Horridge, 1954*b*) that at each beat a single impulse in the giant fibre system precedes the contraction wave. This means that a separate conducting system must be found for the transmission of excitation which is not accompanied by a contraction wave.

2. In *Geryonia* (Hydromedusae) two different kinds of excitation can be transmitted *simultaneously* in different directions over the sub-umbrellar epithelium (Horridge, 1955).

3. In the ephyra larva of *Aurellia* the beat and the feeding response can

occur separately. One affects the whole bell or all unoccupied arms momentarily, the other only one arm for long periods.

4. Again in the ephyra, certain areas transmit excitation that leads to one response but not to the other; other areas do so for either response.

Any one of these points requires something more than the classical concept of one net of nerves. Two histologically distinct nets have now been presented and have been identified with the two conducting systems. This has led to the probability of a double motor innervation and has provided a basis for the interpretation of the neurone structure of the marginal ganglia.

Many earlier observations on large Scyphozoa have indicated an acceleration or inhibition of the rhythm following stimulation at points far from the marginal ganglia. A reflex control of the beat was emphasized by Jordan (1912). The action of the diffuse nerve-net on the ganglion, as shown in the adult *Aurellia* by the initiation of a beat by a marginal tentacular wave, recalls the organization of a reflex arc, but the input, far from being a sensory nerve, is part of the diffuse nerve-net which has motor connexions. It also seems that the rhythm of the beat is not entirely spontaneous as has often been supposed, but that it is modified by sensory excitation from all parts of the bell; the inhibition of the rhythm by a long-lasting excitation in the diffuse net is particularly well marked in the ephyra.

In this paper the physiology of the nervous system of the ephyra has been shown to be more similar to that of higher animals than has formerly been considered likely in any coelenterate. However, this example is not an isolated, curious case, but is typical of its group in both the structure and activity of the nerve-nets. It is not yet clear how far these conclusions can be extended to other coelenterate groups, but most medusae, at least, have a characteristic organization of two overlying but distinct nerve-nets which meet and interact in the marginal ganglia.

I should like to thank Mr. F. S. Russell and the members of his staff at the Marine Laboratory, Plymouth, for their kind assistance and for providing material from the aquarium, and also I should like to thank Dr. F. S. J. Hollick for a helpful criticism of the manuscript.

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Neurosecretory Cells of the Central Nervous System of the Crab, *Paratelphusa hydrodromous*

By R. PARAMESWARAN

(*From the Department of Zoology, University College, Trivandrum, S. India*)

With one plate (fig. 1)

SUMMARY

Three types of neurosecretory cells, designated A, B, and C, occur in the central nervous system of *Paratelphusa hydrodromous*. They are distinguishable by their sizes, the nature of their nuclei, and the secretory products in the cytoplasm. Their distribution and neurosecretory activity are recorded in this paper. All the three types occur in the thoracic ganglion, while the brain and tritocerebral connective ganglia contain only the A and B types. Examined alive under the phase-contrast microscope, the conspicuous A-cells reveal the presence of numerous tiny granules and dark spheroids in the cytoplasm. These spheroids are stainable by neutral red. In Golgi preparations the spheroid walls become black. These are sudanophil and probably represent lipochondria. The spheroids contain phospholipid and vitamin C. The granules of the cytoplasm probably represent the mitochondria.

The brain and thoracic ganglia contain within them a rich network of delicate capillaries which surround individual neurosecretory cells as well as groups of cells. The secretory products are possibly discharged directly into the blood.

INTRODUCTION

THE occurrence of neurosecretory cells, i.e. neurones having a special glandular activity, has been noticed in the crustacea by many workers during recent times (Scharrer and Scharrer, 1954). Much of the work has been concerned with the sinus gland and X-organ complex of the eye-stalk. The first attempt at mapping the neurosecretory cells of the central nervous system of a crab was made by Enami (1951*a*) on *Sesarma*. Later Bliss and Welsh (1952) gave a map for the brain of *Gecarcinus*, and subsequently Bliss, Durand, and Welsh (1954) for the crayfish, *Cambarus*. Among the Brachyura, the works of Enami (1951*b*) and of Matsumoto (1954*a*) are the most important. They have not only given detailed accounts of the distribution and structure of the neurosecretory cells, but also have attempted to elucidate their functional roles (Enami, 1951*a*; Matsumoto, 1954*b*).

In this paper the distribution and nature of the neurosecretory cells in the central nervous system of *Paratelphusa hydrodromous* (Herbst) is described. A preliminary note on the presence of neurosecretory cells in this crab was reported in a previous note by the author (Parameswaran, 1955).

MATERIAL AND METHODS

Paratelphusa hydrodromous is a common fresh-water crab found in paddy fields. The material for the present study consisted of males and females ranging from 10 to 40 mm in carapace length.

The animals were dissected in Ringer's solution and the parts of the central

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nervous system were quickly removed and fixed in Bouin's, Helly's, an Heidenhain's Susa fixatives. Of these, Bouin and Susa gave the best results Paraffin sections were cut at 6μ and stained in Gomori's chrome-alum haematoxylin-phloxin, Mallory's triple stain, Heidenhain's Azan, an Masson's trichrome. Transverse, horizontal, and sagittal sections were mad to reconstruct the distribution of the neurosecretory cells.

Living cells were examined under the phase-contrast microscope as well a after supravital staining.

Besides the above methods, the following special techniques were used t study the structure of the A-cells:

For the study of the spheroids

(*a*) Aoyama's method (1929) for the Golgi apparatus;
(*b*) Weigl's Mann–Kopsch method (1910);
(*c*) Thomas's method (1947) of studying the Golgi apparatus by treatmen in 1% osmium tetroxide and examination from time to time;
(*d*) Baker's Sudan black method (1944) after fixation in formaldehyde saline;
(*e*) Thomas's method (1948) of colouring paraffin sections with Sudan black;
(*f*) McManus's method (Pearse, 1953) for lipids.

For mitochondria

Material fixed in Altmann's fluid or in 0·75% HCl (Casselman and Jordan 1954) was used, stained either in Hirschler's haematoxylin or in acid fuchsin according to Metzner.

Histochemical tests

(*a*) Baker's (1946) acid haematein and pyridine-extraction tests fo phospholipids;
(*b*) Barnett and Bourne's method (1942) for vitamin C.

OBSERVATIONS

There are three types of neurosecretory cells in the central nervous system of *P. hydrodromous*. They could easily be distinguished from the ordinary nerve-cells by their larger size and nuclei, conspicuous nucleoli, and staining

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FIG. 1 (plate). A, A-cell, showing coarse cytoplasm. The scale represents 20μ.
B, the same, showing small vacuoles in the cytoplasm. The scale represents 30μ.
C, the same, with vacuoles at the periphery of the cell. The scale represents 20μ.
D, B-cells, showing vacuoles. The scale represents 20μ.
E, a group of neurosecretory cells with small blood-vessels round them. The scale represents 60μ.
F, transverse section of the brain, showing the neurosecretory cells round the neuropiles The scale represents 140μ.
G, a group of C-cells. The scale represents 15μ.
H, an Aoyama preparation of the C-cell showing the spheroids blackened by silver. The scale represents 10μ.

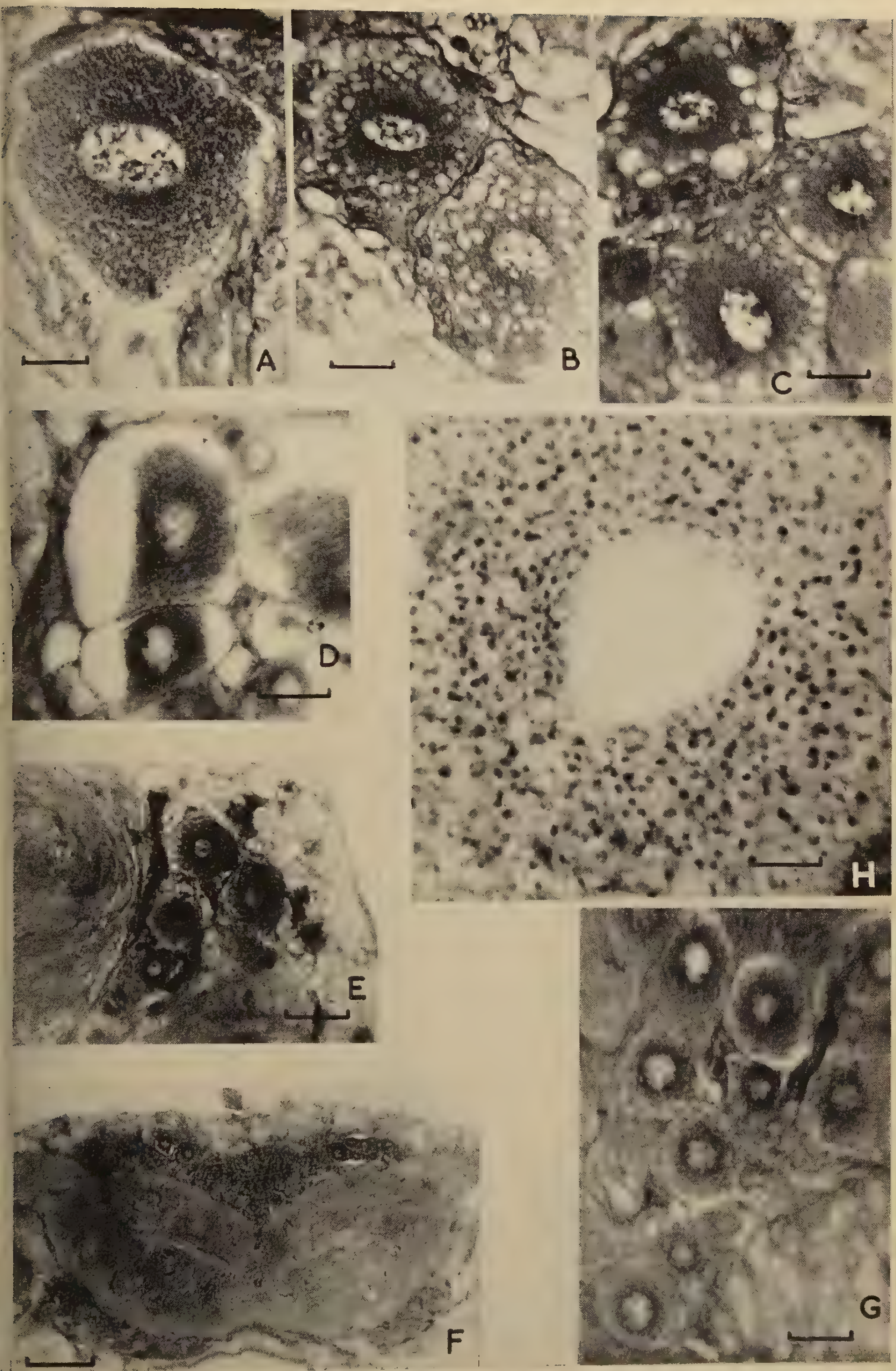

Fig. 1

P. PARAMESWARAN

reactions. Their cytoplasm shows deep blue granules when stained with Gomori's chrome-alum-haematoxylin-phloxin. The granules are stained red or bluish-red in Azan and Mallory and bluish in Masson's. The cells resemble those of *Eriocheir* described by Matsumoto (1954*a*) and are designated by the same names, A, B, and C.

A-cells

These are giant cells. The largest measure 160μ in diameter in the living condition when gently flattened under the cover-glass. They are very similar in detail to the A-cells of *Eriocheir*. They exhibit changes in nuclear form associated with their secretory activity. The cytoplasmic granules are crowded round the flattish nuclei. Many cells show a coarse cytoplasm (fig. 1, A). In many, a number of small vacuoles occur in the cytoplasm (fig. 1, B). In some, the vacuoles are larger and occur only at the peripheral portions of the cell (fig. 1, C). All these may be considered as stages in their secretory activity.

B-cells

These are much smaller than the A-cells, measuring 30–45μ in diameter in the living condition and show a different type of secretory activity. Some of the cells show a coarse cytoplasm with deep blue irregular masses of secretory granules by Gomori's method. Vacuoles are not noticed at this stage. In many others the cytoplasm is perfectly homogeneous, with numerous dark blue granules distributed densely and uniformly so that the cell as a whole appears bluish. In some of these cells one to three large vacuoles are noticed at the periphery (fig. 1, D). No modifications of the nuclei are seen and the aggregations of the secretory granules into one or two large masses at the periphery, as observed by Matsumoto, have not been noticed here.

C-cells

These are identical with the C-cells of *Eriocheir*. They are 30–45μ in diameter. The cytoplasmic granules occur in irregular clumps, denser round the nucleus (fig. 1, G). This cytoplasmic nature is very characteristic of these cells. The nucleus is spherical. Vacuolization is absent.

Distribution

The three types of cells show definite localization in the central nervous system and their distribution is illustrated in figs. 2 and 3, D and G. All the three types occur in the thoracic ganglion, while the brain and tritocerebral connective ganglia contain only the A and B types.

In the brain, the neurosecretory cells surround the principal neuropiles (figs. 1, F and 2). The A-cells are comparatively few and occur anteriorly and laterally on the dorsal side, and laterally and medially on the ventral side. They are generally much smaller than those of the thoracic ganglion, the largest measuring up to 105μ in diameter in the living condition. The B-cells

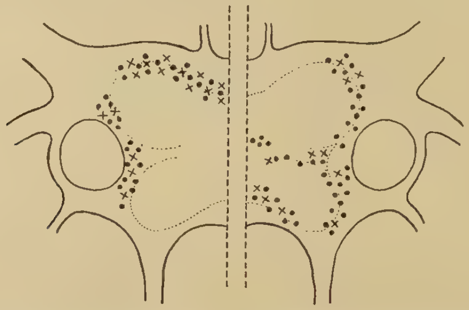

FIG. 2. Diagram showing the distribution of neurosecretory cells in the brain. The cross represent A-cells, the black circles B-cells. Left side dorsal, right ventral.

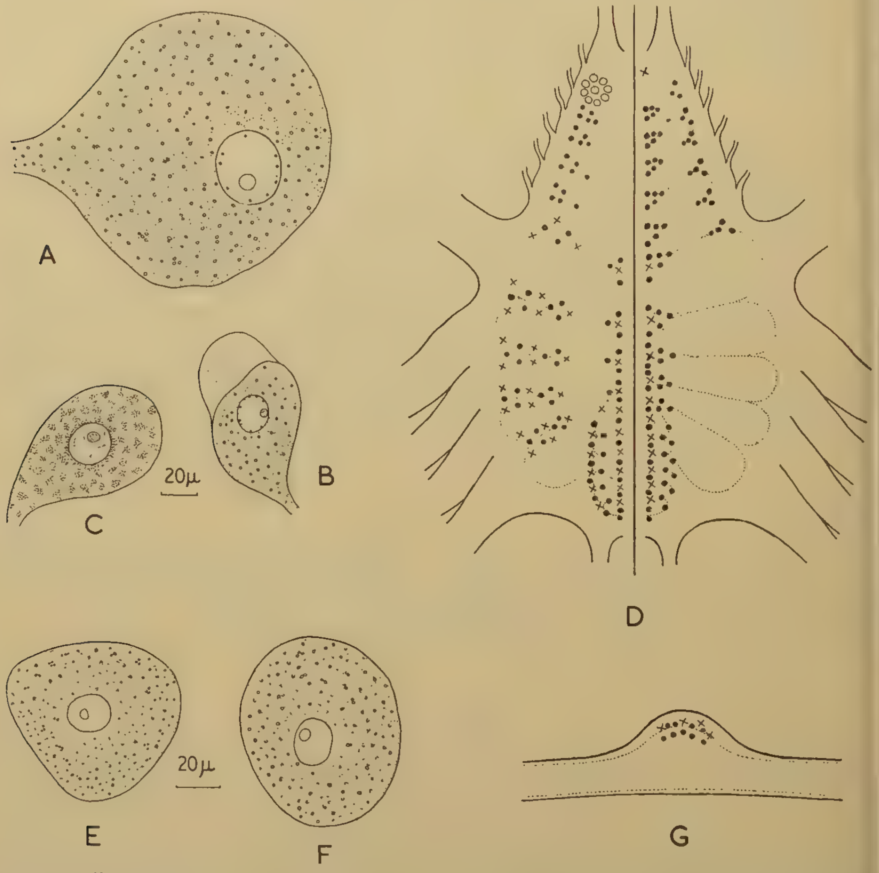

FIG. 3. A, diagram of A-cell as seen alive under phase-contrast. B, diagram of B-cell, ditto c, diagram of C-cell, ditto. D, diagram showing the distribution of neurosecretory cells in the thoracic ganglion. The crosses represent A-cells, the black circles B-cells, the white circles C-cells. The arrangement in the dorsal region is shown on the left, in ventral region on the right. E, Mann–Kopsch preparation of A-cell. F, Sudan-black preparation of A-cell. G diagram showing the distribution of neurosecretory cells in the tritocerebral connective ganglion. (Symbols as in D.)

are numerous and crowded together anteriorly and laterally on the dorsal side, and laterally and medially on the ventral side.

The tritocerebral connective ganglia are a pair of rounded prominences projecting outwards from the oesophageal connectives in front of the tritocerebral commissure. In each ganglion there are laterally a few small A-cells and a number of B-cells (fig. 3, G).

The thoracic ganglion is a large, composite structure formed by the fusion of all the ganglia from the mandibular to the last abdominal segment. The neuropiles representing these ganglia could be distinguished in it. The neurosecretory cell-clusters are always found outside the neuropiles and their axons could be traced into the neuropiles. The A-cells are the most conspicuous elements and are distributed in all parts of the ganglion mainly along the mid-dorsal, mid-ventral, and dorso-lateral regions but are rare in the anterior part (fig. 3, D). Some of the largest A-cells are found towards the posterior part of the ganglion. The B-cells occur in large numbers round the abdominal neuropiles, along the middle part of the ganglion, and in the anterior ventral region. In this latter part they occur unmixed with the A-cells. In all these areas they occur in small groups. The C-cells occur only in the thoracic ganglion and in a restricted area in the anterior region. They form a pair of large, compact clusters between the first and second neuropiles and enveloping their lateral surface. Their axons could be traced into the central mass of the neuropiles.

Living cells

A-cell (fig. 3, A). The nucleus is rounded, oval, or nearly flat. It is situated centrally or slightly towards the abaxonal region. The nucleolus is conspicuous and has a high refractive index. The cytoplasm is densely granular. The granules are very minute and appear grey by positive phase-contrast; some of them show Brownian movement. The cytoplasm also contains numerous spheroid bodies, larger than the granules, but somewhat variable in size. These spheroids are more or less evenly distributed. The rim of each spheroid appears black by positive phase-contrast, and the internal part pale. The granules and spheroids are traceable into the axons. In some cells small vacuoles are seen in the cytoplasm. Granules showing Brownian movement are seen in these vacuoles.

B-cell (fig. 3, B). The nucleus is large and spherical, with a conspicuous nucleolus. Some of the chromocentres touch the nuclear membrane. The cytoplasm is densely granular. The spheroids are small, almost black by phase-contrast, and more or less uniformly distributed. In the abaxonal region of the cell there is a large, clear vacuole that tends to become separated from the rest of the cell under the pressure of the cover-glass. Brownian movement is seen in the granules of the cytoplasm and vacuole.

C-cell (fig. 3, C). The nucleus is large, spherical, and placed centrally. It has a conspicuous nucleolus and irregular chromocentres. The cytoplasm has a frothy appearance and shows numerous tiny granules and very small spheroids, dark by phase-contrast. These are found in irregular masses distribut throughout the cytoplasm, with a dense concentration round the nucle membrane. Vacuoles are not present.

In supravital staining, neutral red gave the best results (one drop of 0·1 neutral red to 15 drops of Ringer's solution). In the A and B cells it stains t spheroids in 5–10 minutes. They appear as irregular reddish dots. In t C-cells the cytoplasm has the frothy appearance and contains clusters reddish bodies in the meshes. These are crowded round the nucleus.

Discharge of neurosecretory material

It is possible that the discharge of the neurosecretory products occu directly into the blood in the rich network of minute vessels surrounding t cells (fig. 1, E). An axonal transport into storage centres may also be present, secretory granules accumulate among the neuropiles along the pathways the axons.

Structure of the A-cell

The nature of the spheroids and granules of the cytoplasm of the A-cell w studied by the standard techniques. In the classical Golgi techniques Aoyama and Mann–Kopsch, the spheroids become enveloped in a deposit silver or osmium (figs. 1, H and 3, E). The pictures obtained by both the methods are the same. In the former method, the spheroids often show th characteristic ring-like appearance, with the deposit of silver on their wal only, but many of the spheroids become completely impregnated with silve Sometimes the spheroids tend to accumulate in groups of three or four. I the Mann–Kopsch preparations complete blackening by osmium is obtaine in 5 days. The spheroid wall is thick and black and the interior clear. Th appearance of these Golgi bodies is similar to that of the spheroids as see under phase-contrast.

Thomas's method of slow osmication of fresh tissue in 1% osmiu tetroxide with periodical examination reveals the gradual blackening of th outer rim of the spheroids. Some of the tiny spheroids completely blacke in 6 hours. In 24 hours all except the larger spheroids have fully blackene The picture obtained is the same as in the standard Golgi preparations.

Sections stained in Sudan black by Baker's method show irregular, dee blue bodies in the cytoplasm and these resemble the Aoyama and Mann Kopsch preparations (fig. 3, F). The sudanophil material evidently represent the spheroids. The result was confirmed by using Thomas's modification c the above technique with paraffin sections and also by McManus's method These tests show that the so-called Golgi bodies are lipochondria. Baker' acid haematein test showed that the spheroids contain phospholipid. Pyridin extraction followed by acid haematein confirmed this. Tests for vitamin C gave positive results in the spheroids.

Mitochondria

The numerous minute granules seen in the cytoplasm by phase-contrast ppear to be mitochondria. Metzner's and Hirschler's methods for staining mitochondria show similar granules in the cytoplasm, especially near the acuoles.

DISCUSSION

The neurosecretory cells of *Paratelphusa* closely resemble those of *Eriocheir* n structure and distribution (Matsumoto, 1954*a*). While Matsumoto has described only the cells of the thoracic ganglion, the present work is extended o include the brain and the tritocerebral connective ganglia as well. There are hree types of neurosecretory cells, of which only the A and B types occur in he brain and tritocerebral connective ganglia. The A-cells of these regions re smaller than those of the thoracic ganglion. In *Sesarma*, Enami (1951*b*) has hown the presence of three types of neurosecretory cells of which only one ype (his α type) occurs in the thoracic ganglion.

Living neurosecretory cells of crustacea have been examined by Passano 1954), who studied those of the eye-stalk. In *Paratelphusa* the cells reveal the presence of numerous spheroids and minute granules in the cytoplasm. Both kinds of inclusion are conspicuous under phase-contrast. The spheroids re stainable by neutral red. In standard Golgi preparations a picture similar o that seen under the phase-contrast is obtained and this suggests that the Golgi bodies represent the spheroids (lipochondria) seen in life. These are rgentophil, osmiophil, and sudanophil. Such spheroids have been described n the neurosecretory cells of insects like *Chaetodacus cucurbitae* (Nayar, 1954) nd *Iphita limbata* (Nayar, 1955), and in the motor neurones of *Locusta migratoria* (Shafiq, 1953 and 1954). The lipochondria contain phospholipines and vitamin C. The minute granules of the cytoplasm seen in life probably represent the mitochondria.

Though nothing definite could be said regarding the way in which neurosecretory material is discharged from the cells, there is evidence that the secretory products are discharged from the periphery of the cells into the very small blood-vessels surrounding them in the brain as well as in the thoracic ganglion. This mode of discharge has been suggested by Matsumoto (1954*a*). Enami (1951*b*) and Bliss and Welsh (1952) suggested an axonal transport of neurosecretory material into storage centres like the sinus gland, whence they diffuse into the blood. In the vertebrates, Scharrer and Scharrer (1954) have shown that the discharge of neurosecretory products occur into the capillaries both directly and through the axons. The present work lends support to the view of Matsumoto (1954*a*).

ACKNOWLEDGEMENTS

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The Structure of the Corpus Allatum of *Iphita limbata* (Hemiptera)

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With one plate (fig. 1)

SUMMARY

The corpus allatum of *Iphita limbata* Stal. (Pyrrhocoridae, Hemiptera) is an unpaired, syncytial gland, with nuclei of two sizes. The allatic nerve transmits neurosecretory colloids into the gland. The recurrent nerve adjacent to the allatic nerve also contains neurosecretory products. The cytoplasm of the gland contains granular mitochondria which show a tendency to aggregate into clusters or rings and chains, and numerous tiny spheroids of variable size forming the lipochondria, which are sudanophil, argentophil, and generally osmiophil. Vacuoles are present in the cytoplasm; these are surrounded by spheroids. The cytoplasmic content is rich in phospholipids. The gland is larger in the female. It gradually enlarges as the eggs develop and reaches its maximum size in the gravid female.

INTRODUCTION

THE physiological significance of the corpus allatum, as an endocrine gland of insects, has been investigated by various authors (see reviews by Scharrer (1952*a*), Bodenstein (1953), and Wigglesworth (1954)); but comparatively little is known about its detailed structure and cytology. A general account of the gland has been published by Nabert (1913), who describes it in the different groups of insects. A comparative study of the retrocerebral endocrine organs of insects has been published by Cazal (1948), who deals with the cytological details of the corpora cardiaca and allata besides describing them in the various representative types of the group. He classifies the corpus allatum into four types, based on the cellular nature of the gland. Mendes (1948) has dealt with the cytology of the corpora allata of the grasshopper *Melanoplus*; he gives details of the various stages of development. The variability in size of the gland in the two sexes has been studied by Palm (1949).

Many of the studies of the corpora allata in recent years deal with the histophysiological nature of the gland in relation to the neurosecretory system. The products of the neurosecretory cells of the brain, stainable by Gomori's chrome alum haematoxylin / phloxin, pass along their axons to the corpora cardiaca and then to the corpora allata. This transport of neurosecretory material to the corpora cardiaca and the allata has been seen in *Leucophaea* by Scharrer (1952*b*), in *Bombyx* by Arvy, Bounhiol, and Gabe (1953), in Plecoptera by Arvy and Gabe (1954), in *Calliphora* by E. Thomsen (1954), and in Hymenoptera by M. Thomsen (1954).

The present study forms part of an investigation of the structure and functions of the neurosecretory system of the plant bug *Iphita limbata* Stal.

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(Pyrrhocoridae, Hemiptera). A preliminary note on this has been publishe by the author (Nayar, 1953).

MATERIAL AND METHODS

The corpus allatum was dissected out from the adult insect collected fresh in the field. When the pronotum and the dorsum of the head are removed an the head stretched forwards by pulling the rostrum, the aorta and brain ar revealed. Under the stereoscopic binocular (×40) the corpus allatum wa removed along with a short bit of aorta and then manipulated in a drop o Ringer on a slide. The material was then fixed or treated as required.

Entire sections of the head and thorax were cut to find out the relationshi between the different parts of the neurosecretory system. The fixed materia was processed by Peterfi's methyl benzoate / celloidin method and paraffi sections 5μ thick were cut.

The following methods were used:

1. *For general anatomy and histology*: Bouin-fixed material stained i Gomori's chrome alum haematoxylin / phloxin; Smith-fixed material (B. G Smith's fluid) stained in Heidenhain's iron haematoxylin.
2. *For study of living cells*: Cells studied under phase contrast microscope cells supravitally stained in neutral red, dahlia violet, and Janus gree (0·01%).
3. *For mitochondria*: Metzner and Krause's (1928) method and Hirschler' (1918) method after fixation in Altmann's or Helly's fluid.
4. *For lipochondria*: Osmium impregnation methods of Weigl (1910) an Kolatchev (1916) after fixation in Champy's fluid, and the silver impregnatio technique of Aoyama (1929); Baker's (1949) Sudan black method.
5. *Histochemical methods*: Brachet's (1953) method for nucleic acids afte fixation in Serra's mixture; Feulgen and Rossenbeck's reaction; Baker' (1946) acid haematein test for phospholipids; Cain's (1947) Nile blue metho for acidic and neutral lipids; and Millon's reagent for proteins.

OBSERVATIONS

The corpus allatum is a median, composite gland, situated just behind th brain and posterior to the paired, tiny corpora cardiaca. It is attached to th ventral edge of the anterior end of the aorta. The gland appears brownish under the stereoscopic binocular. It is supplied by a conspicuous trachea o each side, which springs from a longitudinal trunk running along the lateral edge of the aorta. This branch runs towards the posterior two-thirds and divides into three tracheoles giving rise to finer tracheoles supplying the gland.

The gland is invested by a firm, thin, transparent membrane. Anteriorly the allatic nerves forming a closely packed set of fibres connect the gland with the corpora cardiaca.

In the last instar nymph the corpus allatum measures about 364 to 390μ in diameter. Here the gland is disk-like and circular in outline. In the adult male, the gland measures about 390μ in diameter; sometimes it is even smaller, measuring about 325μ. In the newly moulted adult female which is unmated and 1 day old, it measures about 351μ from side to side and 260μ antero-posteriorly. As the female begins mating and the eggs develop, the corpus allatum enlarges. The mating female, without swollen abdomen, has a corpus allatum of diameter 520μ and length 390μ; in the gravid female with swollen abdomen the corpus allatum reaches the maximum size, being large, flattened, and two-lobed, with a transverse diameter of 910μ and a length of 780μ. The relative size of the two lobes varies: sometimes they are nearly equal.

After oviposition the corpus allatum remains swollen for a few days and then tends to shrink in size. After a week it measures about 520μ in diameter and 390μ in length.

The syncytium

The freshly dissected corpus allatum does not show any cellular limits; and no cell limits have been observed in any fixed preparation. Special studies were made by using an adaptation of Recklinghausen's method (Nayar, 1954), which revealed that the gland has no distinct cell borders. It may be described as syncytial.

In general routine preparations (fig. 1, A) the nuclei often segregate from a granular mass which probably represents a part of the secretory content of the gland. When stained in Gomori's chrome alum haematoxylin / phloxin, these granules are blue, resembling the neurosecretory granules and colloids of the cerebral neurosecretory cells and the corpora cardiaca. The tendency of the nuclei to clump to one side, when ordinary routine fixatives are used, is marked in the corpus allatum of *Iphita*. A number of vacuoles are also present in the cytoplasm.

Two types of nuclei are present in the corpus allatum. Large, transparent ones, having an ovoid or (rarely) spherical shape, are especially numerous towards the peripheral parts of the gland. These nuclei measure on an average about 14 to 19μ in length and 10 to 14μ in diameter, in the female. A large number of smaller, rounded nuclei, which have a higher refractive index, are present in fairly large numbers in the central region of the gland. These measure about 8·5 to 11μ in diameter. In methyl green / pyronin solution, both kinds of nuclei are stained green. The chromatin of both types of nuclei is Feulgen-positive.

Ground cytoplasm

The cytoplasm of the corpus allatum is basiphil; it is coloured pink in methyl green / pyronin. The basiphilia is due to pentose nucleic acids as prior treatment with HCl prevents the development of the colour.

Neurosecretory material

This stains bright blue by Gomori's chrome alum haematoxylin / phloxin. It is traceable through the axons of the neurosecretory cells of the brain. It accumulates as dark blue irregular masses in the corpus cardiacum and corp allatum. In the latter, the neurosecretory material is seen coming throug the allatic nerve (fig. 1, B and C) from the corpus cardiacum. It appears in th form of clumps entering the body of the corpus allatum. Lying close to th allatic nerve and probably connected to it is the recurrent nerve of the stomato gastric system. This nerve also shows an accumulation of neurosecretor material along its course.

Mitochondria

The cytoplasm shows granular mitochondria which have a tendency t clump together to form small masses or rings of granules. Sometimes a succession of such rings is visible in certain regions. The living tissue under th phase-contrast microscope reveals the presence of minute granules showin active Brownian movement, and appearing dark when properly focused. N special staining of the granules by Janus green was noticed.

Lipochondria

Lying in the cytoplasm are spheroids of variable size, which appear as ver minute droplets. Some of them show well defined black rims and a transparen medulla under the phase-contrast microscope. Others, including the smalle ones, appear dark throughout (fig. 1, D).

The spheroids are stained by neutral red, and then give a mottled appearance to the cytoplasm. The size of the coloured spheroids varies; thos measuring about 0·3 or 0·4μ in diameter are numerous, others measure fro 0·5 to 0·7μ; a few large ones measure up to 1·4 or even 2μ or more in diameter The spheroids are seen in the cytoplasm near or sometimes around rounde vacuoles. These vacuoles are not coloured by neutral red.

No selective staining was obtained with dahlia violet.

In 'Golgi preparations' made by Aoyama's method, the spheroids are blackened by silver (fig. 1, E). A similar picture is obtained by osmiu methods. Weigl's method was more reliable than Kolatchev's. Simple haematoxylin staining after Champy-fixation rendered the spheroids blue.

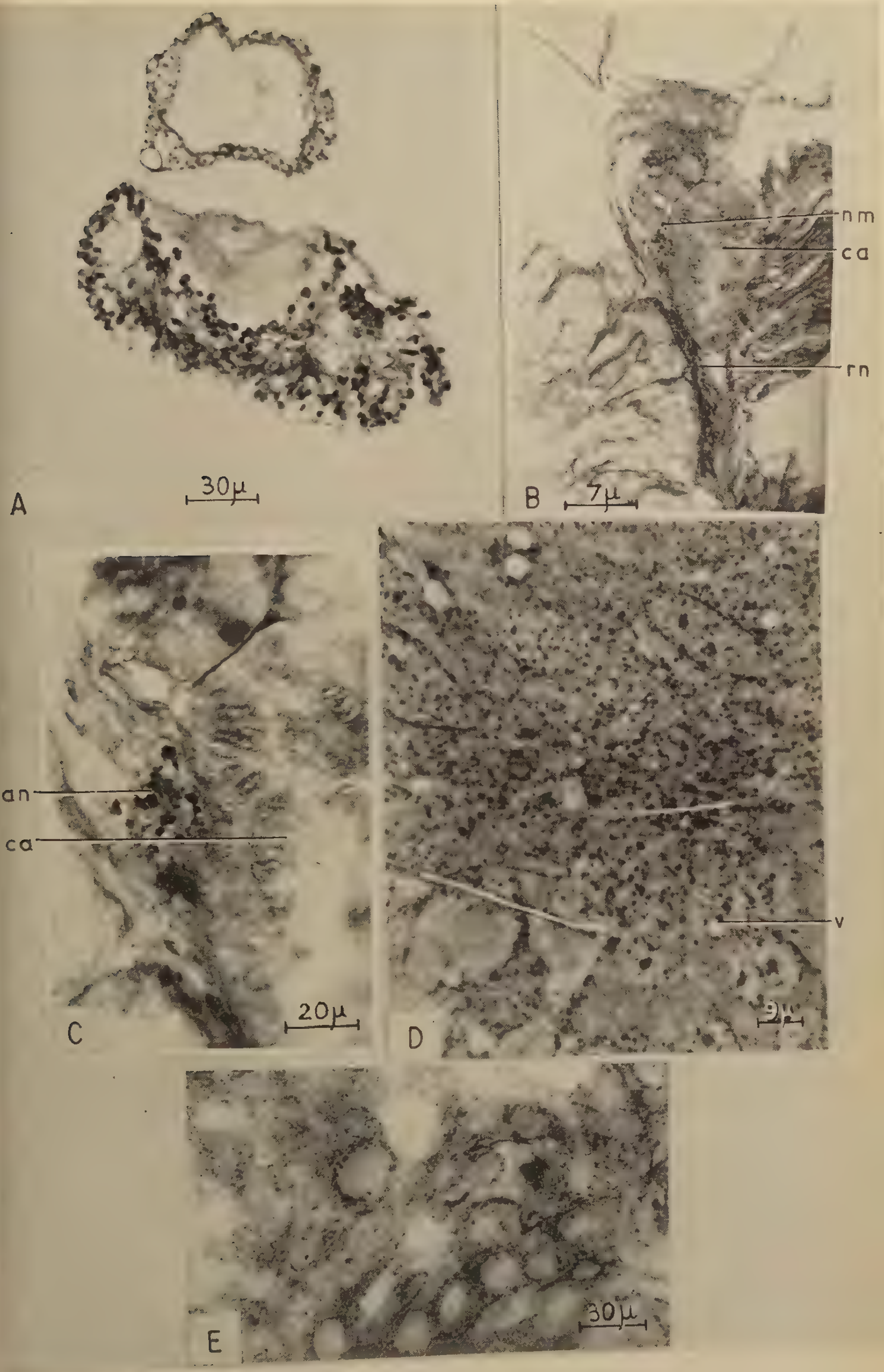
FIG. 1 (plate). A, section of the corpus allatum of the last instar nymph of *Iphita limbata*, t show the tendency of the cytoplasmic inclusions to clump to one side. Fixed in Smith's fluid and stained in iron haematoxylin. The black rounded bodies are the nuclei. The aorta is see lying above the corpus allatum. A number of vacuoles are seen as irregular spaces in the cytoplasm.

B, longitudinal section of the female to show neurosecretory pathways. The neurosecretory material (*nm*) enters the corpus allatum (*ca*) through the allatic nerve. The recurrent nerve (*rn*) running posteriorly also contains the colloids. Fixed in Bouin's fluid and stained in Gomori's chrome alum haematoxylin / phloxin.

C, higher magnification of B to show the accumulation of the secretory colloids at the tip of the allatic nerve (*an*) entering the corpus allatum (*ca*).

D, part of the living corpus allatum under the phase contrast microscope. The spheroids appear as black particles and some as blackish rings. Spheroids are seen associated with rim of clear vacuoles (*v*). Two rounded nuclei are seen at the bottom towards the right.

E, spheroids of the corpus allatum revealed by Aoyama's technique. The vesicular bodies are the nuclei.



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Gradual osmication as recommended by Thomas (1948) was not very useful. In the earlier stages the gradual darkening of the spheroids was discernible up till about 24 hours, but later the whole region darkened, hiding the details. A specific blackening did not occur.

Baker's Sudan black method showed the nature of the spheroids very well. They appeared as dirty brownish-black spheres of varying sizes, arranged around the larger vacuoles, recalling the condition in the living cells.

Vacuoles

In the cytoplasm are numerous vacuoles, most of them small (about 1·5 to 3μ in diameter); the larger ones measure up to 5 or 6μ in diameter. Under phase contrast they appear transparent. The spheroids are seen clustered round or adjacent to these vacuoles.

Histochemical notes

Baker's acid haematein test shows that the cytoplasmic contents—both spheroidal and granular—are rich in phospholipid. This is confirmed by the pyridine extraction test.

With Cain's Nile blue, most of the lipochondria stain blue. Red droplets (neutral lipids) are also present in the cytoplasm.

When treated with Millon's reagent the cytoplasm is coloured red, but the cytoplasmic inclusions are not specifically coloured.

DISCUSSION

The general histological nature of the corpus allatum in Pyrrhocoridae has been described by Nabert (1913) and in various Hemiptera by Cazal (1948). In *Iphita limbata* the gland may be described as a modified parvocellular type, according to Cazal's classification.

The neurosecretory pathways have been described both from histological and experimental points of view, in recent years. In *Iphita limbata* the supply of neurosecretory products to the corpus allatum resembles the condition described by various workers in other groups of insects. The presence of neurosecretory material in the recurrent nerve of the stomatogastric system has been previously observed in ligated living nerves of *Calliphora* by E. Thomsen (1954).

The lipochondrial and mitochondrial components of the cytoplasm have been described in the corpus cardiacum of the *Locusta migratoria* by Nayar (1954). The abundance of the lipochondria and the vacuoles in close association with them probably indicate the secretory activity in the cells. In routine preparations, the vacuolation in the gland has been noticed by various authors (Vogt, 1941, 1943; Thomsen, 1942; Cazal, 1948).

The lipochondria are stained by neutral red supravitally and are sudanophil and argentophil; but osmium is generally deposited between and round them. Baker's Sudan black and Aoyama's method reveal the spheroids almost as they occur in living cells. Thomas (1954) has shown that deposition of osmiu occurs sometimes in a non-specific and diffuse manner in the neurons o *Lumbricus.*

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The Haemocytes and Connective Tissue Formation in an Insect, *Rhodnius prolixus* (Hemiptera)

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SUMMARY

The amoebocytes, which form the most abundant type of blood-cell in *Rhodnius*, contain rounded, oval, or rod-shaped inclusions which stain by the periodic acid / Schiff method. These are believed to be neutral mucopolysaccharides. The amoebocytes apply themselves to the basal membranes, which likewise are PAS-positive, and appear to contribute to these membranes by discharging their contents. They insinuate themselves into the developing muscles and give rise to the sheaths of connective tissue by which the muscle fibres are surrounded. And they collect around deposits of injected Indian ink, producing similar sheets of PAS-positive material, presumably mucopolysaccharide.

The chitinous endocuticle is PAS-negative (except in a few special regions such as the neck and the conjunctival membranes of the limbs). It becomes positive during digestion by the moulting fluid. The tracheae react similarly.

Other PAS-positive structures which are not produced by the amoebocytes are the striated border of the mid-gut, the basement membrane of the gut, the perilemma around the ganglia and nerves, and the ground substance within the ganglia.

INTRODUCTION

IN a recent paper on the role of the haemocytes during growth in *Rhodnius* (Wigglesworth, 1955), it was suggested that the most abundant type of blood-cell, the phagocytic 'amoebocyte', plays an essential part during the initial stages of moulting when the moulting hormone is being secreted. These cells apparently intervene between the neurosecretory cells of the brain and the thoracic gland.

An obvious possibility was that the product of the neurosecretory cells, after being transferred to the corpus cardiacum and discharged into the blood, might be transported to the thoracic gland by the amoebocytes. Sections of the brain and corpus cardiacum, stained with the chrome-haematoxylin method of Gomori (1941), were therefore prepared.

As in other insects the neurosecretory cells were found to be filled with blue-black deposits; material with the same staining properties was present between the cells in the medial part of the corpus cardiacum (fig. 1); and around the corpus cardiacum and adjacent tissues there were amoebocytes with round, oval, or rod-shaped inclusions which stained in the same way (fig. 1, c).

The chrome-haematoxylin stain is in no way specific for the neurosecretory product; other structures staining blue-black in these same sections are the

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perilemma around the ganglia and nerves, the material separating the neurone within the ganglia, the finer tracheal branches, the basement membranes, an the connective tissue sheaths around and between the muscle fibres.

Sections stained with fuchsin-paraldehyde (Gabe, 1953) show the neuro secretory cells, the inclusions in the amoebocytes, and the connective tissu

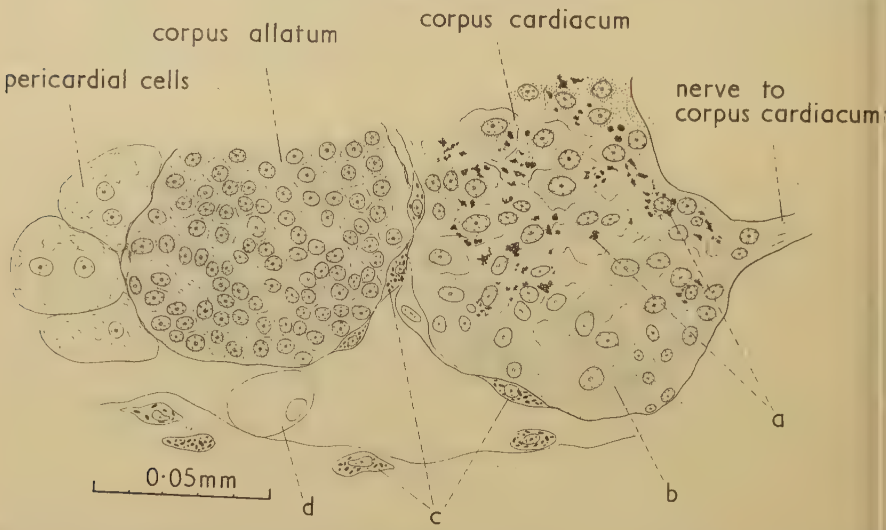

FIG. 1. Horizontal section through the postcerebral organs in the 5th-stage larva of *Rhodnius* chrome-haematoxylin of Gomori. A, intercellular deposits in the corpus cardiacum staining blue-black. B, lateral part of corpus cardiacum without dark staining deposits, apparently producing intrinsic secretion. C, amoebocytes containing blue-black inclusions. D, lipocyte o small fat-body cell.

structures all staining deep red (table 1). On the other hand, in sections stained with Masson's trichrome (method of Foot), whereas the neurosecretory substance stains red with the fuchsin, the inclusions in the amoebocytes, and the connective tissues, stain green.

TABLE 1

Stain	*Neurosecretory product*	*Amoebocyte inclusions*	*Connective tissues*
Chrome-haematoxylin . .	blue-black	blue-black	blue-black
Fuchsin-paraldehyde . .	deep red	deep red	deep red
Masson's trichrome . . .	red	intense green	intense green
PAS	—	+	+

So far as they go these staining results suggest that the inclusions in the amoebocytes may be the precursor substance of the connective tissues and basement membranes, and that the amoebocytes may be concerned in the formation of these structures. This was the view put forward by Lazarenko (1925). The object of the present work was to reinvestigate this possibility.

METHODS

The role of the haemocytes in connective tissue formation has been studied (i) by observing the behaviour of the blood-cells at all stages of the moulting process, and (ii) by using the periodic acid / Schiff method (after removal of glycogen with ptyalin) as a more specific stain for the substance of the connective tissues. Although the PAS test is given by a wide variety of substances (Pearse, 1953), the PAS-positive connective tissues of insects are presumably composed of some mucopolysaccharide, and on this assumption they will be referred to as such throughout this paper. Attempts to characterize the substance further by the use of toluidine blue after various treatments (Pearse, 1953) gave inconclusive results.

For the general study of the haemocytes during moulting, the abdomen has been cut along the margins and the isolated tergites and sternites with the adherent fat body and haemolymph, fixed in Bouin's or Carnoy's fluids, stained with the ferric trioxyhaematein of Prenant, and mounted whole. For the staining of connective tissues the insects were fixed in neutral formaldehyde or in Carnoy and sections of the abdomen and thorax stained by the periodic acid / Schiff procedure after treatment with filtered saliva, and counterstained with light green.

The haemocytes in Rhodnius

A short description of the haemocytes in *Rhodnius* has been given elsewhere (Wigglesworth, 1955). For the most part these cells are not floating freely in the circulating haemolymph but are loosely adherent to the basement membranes, often in large temporary aggregations. As a result, the numbers of cells in blood withdrawn from the body cavity are exceedingly variable: usually the first drop contains a fair number of cells, but subsequent drops very few. The number of free cells can be increased if the insect is immersed in water at 55° C for 5 minutes (Yeager, 1938), but the blood-cell count is still equally variable and probably much below the true figure.

The following cell types are recognized: (i) proleucocytes, the multiplying precursor forms (fig. 2, J); (ii) amoebocytes (fig. 2, A–I); (iii) oenocytoids (fig. 2, K); (iv) lipocytes (fig. 1, D); (v) large granular cells; (vi) large non-granular cells (fig. 2, L). Types (v) and (vi) are relatively few in number; they are only feebly phagocytic and contain no inclusions. The oenocytoids (iii) are rounded or oval disk-shaped cells, non-phagocytic, with homogeneous cytoplasm and without pseudopodia. The lipocytes (iv) do not seem to differ from small detached fat-body cells.

The amoebocytes (ii) are much the most abundant type: in drawn blood they are about ten times as numerous as the oenocytoids, which come second in abundance. They are highly pleomorphic: rounded, spindle-shaped, irregular, or spread out in an attenuated form so as to merge imperceptibly with the basement membrane. When examined in the fresh state they show numerous filamentous pseudopodia (Wigglesworth, 1955, fig. 5). They are active phagocytes. This is the only type with which this paper is concerned.

The inclusions in the amoebocytes, staining with chrome-haematoxylin fuchsin-paraldehyde, and the light green in Masson's trichrome, have alread been noted. In the fresh state these inclusions can be seen as clear glass droplets which stain supravitally with gentian violet, taking on a slate-blu tint. They stain pale pink or mauve with toluidine blue; they are only faintl stained with Prenant's ferric trioxyhaematein and with the bromophenol blu

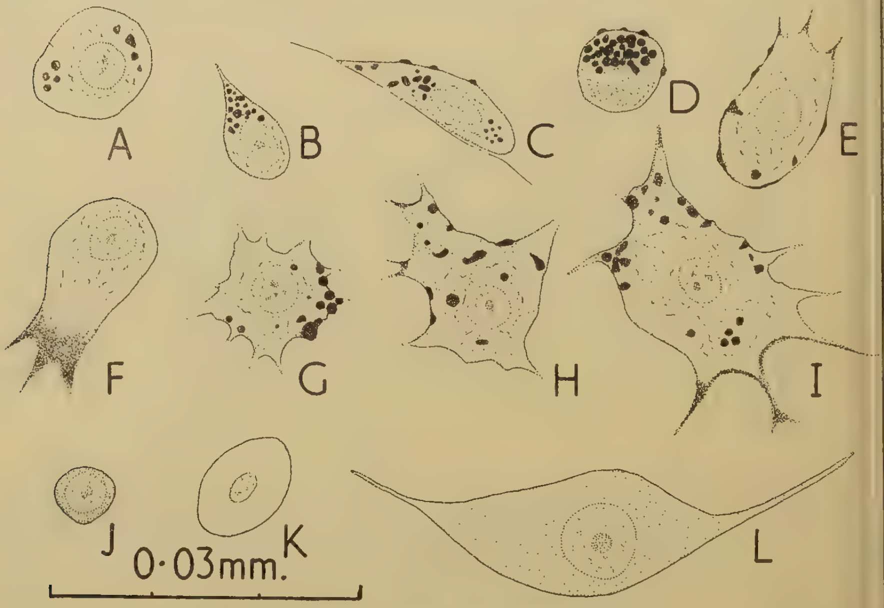

FIG. 2. Haemocytes below the basement membrane of the epidermis in 4th-stage larva a 7 days after feeding: PAS and light green. A–I, amoebocytes (description in text). J, proleucocyte. K oenocytoid. L, large non-granular spindle-shaped haemocyte.

stain for protein (Mazia and others, 1953). They do not stain with Sudan black B nor with osmium tetroxide, nor do they give an argentaffin reaction They give a strongly positive PAS reaction unchanged by treatment with ptyalin, but this reaction is not so intense as in the glycogen deposits of the epidermis or fat-body cells. The inclusions do not stain with alcian blue (Pearse, 1953). They are not strongly basiphil: after treatment with methylene blue at pH 4·0 the cytoplasm of the amoebocytes stains strongly, but the inclusions are unstained. They are presumably composed of neutral mucopolysaccharide material of some kind.

Within a day after feeding, in the 4th-stage larva, many proleucocytes can be seen undergoing mitosis. Multiplication continues, and by 3 days after feeding the amoebocytes are becoming very numerous. Besides the mucopolysaccharide inclusions a few contain colourless vacuoles. By 4 days after feeding the amoebocytes are abundant; many are enlarged and are now filled with the clear vacuoles which seem to be connected with the beginning of moulting (Wigglesworth, 1955). Mitosis in the haemocytes continues until at least the ninth day after feeding: they become increasingly numerous as moulting proceeds.

The formation of connective tissue

At about 6 days after feeding, when mitosis in the epidermis is at its height, the haemocytes are often applied in great numbers to the basement membrane below the epidermis. Fig. 2, A–I shows some of the forms which the amoebocytes assume at this time. A and B are very common types with a relatively small amount of mucopolysaccharide in the cytoplasm. C is a type frequently seen in side view on a nerve or trachea. D is a less common type with extensive massed deposits of mucopolysaccharide. It can be noted in C and D (and the same can be seen in fresh unstained cells) how the deposits seem to be in process of extrusion on to the surface of the cell. E shows a not uncommon condition where nearly all the mucopolysaccharide seems to be extruded on to the surface of the cell and to be merging with the basement membrane. F is an extreme form in which there is only some diffuse PAS staining at one end of the cell, apparently fusing with the basement membrane. G, H, and I represent a very common type in which the cell is spread out in stellate form on the surface of the basement membrane; here again some of the deposits seem to be in process of extrusion and in places seem to merge with the basement membrane, which has the same staining properties with PAS. (This figure contains for comparison a proleucocyte (J), an oenocytoid (K), and a large spindle-shaped non-granular cell (L); all these are devoid of mucopolysaccharide inclusions.)

The general impression to be obtained from these observations is that the amoebocytes are contributing material to thicken the basement membrane. The same impression is gained from observing the muscle insertions. Fig. 3, A shows the attachment of a small muscle to an apodeme in the thorax. Amoebocytes are aggregated around the insertion and among them are free droplets of PAS-positive secretion which are clearly derived from the haemocytes and which appear to be in process of fusing to form the muscle sheath. This sheath is continuous with the basement membrane below the epidermal cells.

More convincing evidence of the part played by the amoebocytes in forming the connective tissues is afforded by the fact that these cells (and no other blood-cells) insinuate themselves between the muscle fibres; here they become exceedingly flattened and clearly give rise to the connective tissue sheaths by which the fibres are surrounded. Fig. 3, B shows a longitudinal section of a small thoracic muscle in the 4th-stage larva during this process. Fig. 4 shows a transverse section of a dorso-ventral abdominal muscle in the 4th-stage larva, in which all stages in the transformation from amoebocytes to sheaths can be followed.

The deposition of these sheaths is most active in the final stages of moulting when the development of the muscles is complete. It is particularly evident in the thorax of the 5th-stage larva when it moults to the adult, for at this time there is a great development of thoracic muscles. In sections of such muscles, containing 200 fibres or more, almost every cleft where three or four fibre come together is occupied by an amoebocyte laden with mucopolysaccharid inclusions, as in fig. 4. Taken altogether this must represent a very larg population of amoebocytes dispersed through the substance of the muscl Later, when the sheaths are fully formed, cells with discrete inclusions can n longer be seen; perhaps they break down and disappear.

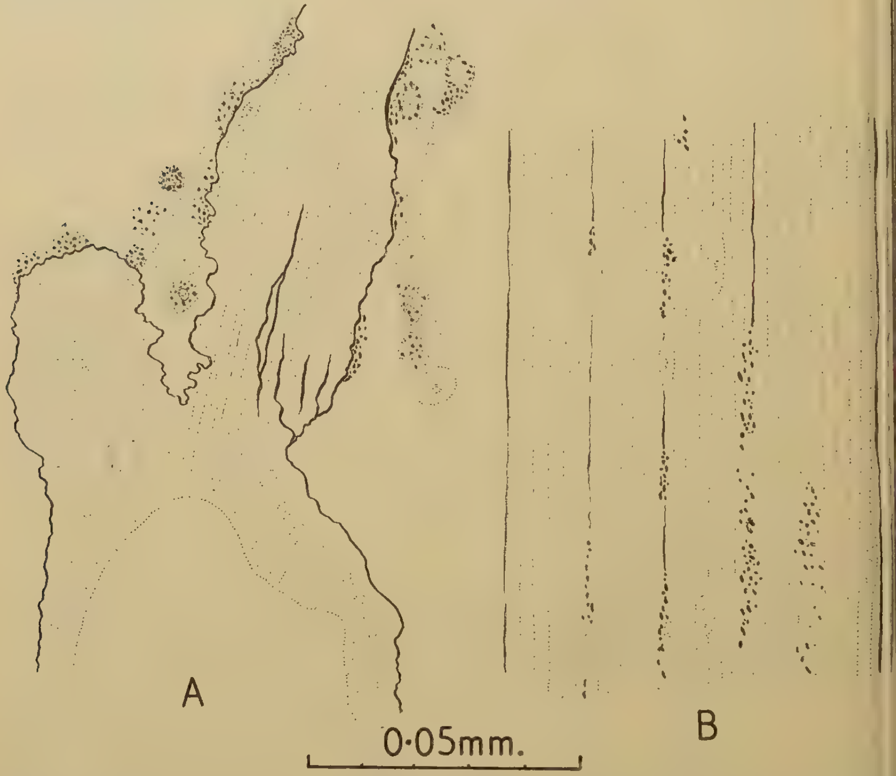

FIG. 3. A, insertion of a small muscle into an apodeme in newly-moulted 5th-stage larva o *Rhodnius*: PAS and light green. Amoebocytes containing PAS-positive inclusions apparentl forming muscle sheath and basement membrane. The blood-cells without inclusions ar oenocytoids. B, longitudinal section of small muscle at same stage as A: PAS and light green Amoebocytes with inclusions flattened between the muscle fibres.

Another circumstance under which the formation of connective tissu sheaths by the amoebocytes becomes evident is after the injection of foreign matter into the blood. The injection of Indian ink is followed by an enormou increase in the numbers of amoebocytes. Eventually, groups of cells laden with the ink become aggregated and surrounded by other haemocytes containing no ink, and these form rather fragile sheaths around the aggregations Fig. 5 shows a section through the margin of one of these deposits. The surrounding haemocytes contain great quantities of 'mucopolysaccharide'; sometimes this appears to be discharged into the intercellular spaces in the form o droplets or strings; sometimes the cells are drawn out into filaments in which the 'mucopolysaccharides' form elongated deposits. Both processes lead to the formation of concentric sheaths of non-cellular material.

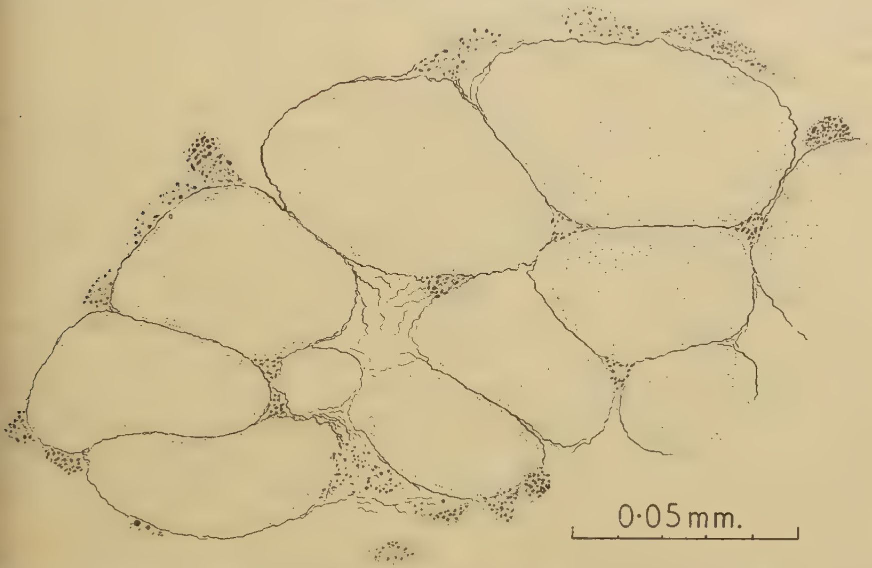

FIG. 4. Transverse section of tergo-sternal muscle in abdomen of 4th-stage larva one day before moulting: PAS and light green. Amoebocytes around and between the muscles giving rise to PAS-positive connective tissue sheaths.

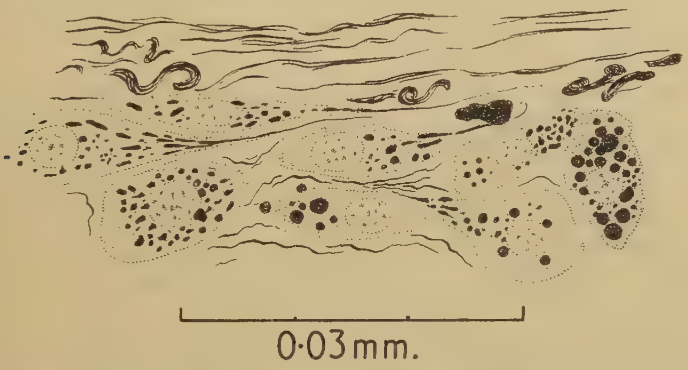

FIG. 5. Connective tissue sheath and amoebocytes around a foreign body. The foreign body (a clump of cells filled with Indian ink) lies at the upper part of the figure but is not included in the drawing. PAS and light green.

Distribution of periodic acid / Schiff staining in Rhodnius

It is certain that the amoebocytes do not have a monopoly in the formation of PAS-staining material. The striated border of the mid-gut, which is strongly positive, must be formed by the epithelial cells. The basement membrane of the intestine is likewise positive; this is separated from the body cavity and the blood-cells by the longitudinal and circular muscle fibres; it seems like that this layer also must be a product of the epithelium. The same applies t the Malpighian tubules.

The contents of the neurosecretory cells are PAS-negative. (Rehm (195 describes the ground substance of the neurosecretory cells in Lepidoptera being PAS-positive; she gives evidence that this reaction is due to boun lipoids.) The sheaths separating the neurones within the ganglia, which ar weakly PAS-positive, are presumably the product of neuroglia cells. And th tough homogeneous 'perilemma' which forms the sheath around the gangli and nerves, and is strongly PAS-positive, is almost certainly a product of th 'perineurium cells' below (Scharrer, 1939). Indeed, it is sometimes possib to see PAS-staining inclusions in these cells. But amoebocytes laden wit 'mucopolysaccharides' can often be seen applying themselves to the surfac of nerves and ganglia; it is possible that they contribute to the formation these sheaths.

The same argument applies to the basement membrane below the epidermi of the cuticle and of the tracheae. This may well have a dual origin, with bot epidermal cells and amoebocytes contributing to it. There must presumabl be a rather close chemical relation between the substance of the basemen membrane and the chitin-protein which is the main product of these cells. I is interesting to note that (contrary to the usual statements in textbooks) th chitinous endocuticle over the greater part of the body in *Rhodnius* does no stain by the PAS method. But there may well be differences in different insect and in different regions of the same insect (cf. Richards, 1952); for in *Rhodniu* the flexible membrane of the neck and some of the conjunctival membrane around the thoracic appendages are sharply marked off from the remainde of the cuticle in being PAS-positive.

During the deposition of the chitinous cuticle there is plenty of glycoge within the epidermal cells, but after this has been removed with ptyalin n PAS-staining material has been observed; whereas at this same time amoebo cytes filled with 'mucopolysaccharide' are applying themselves to the surfac (fig. 2, A–I). During the process of digestion which takes place before the ol skin is shed, the inner layers of the cuticle do stain with PAS, and so likewis does the corresponding layer of the old tracheae. It would seem that durin the digestion of chitin reactive groups are exposed.

Discussion

The connective tissues of insects are generally inconspicuous and littl attention has been given to them. They have been briefly described in *Siali* by Ochsé (1946), who showed that in this insect all the basement membrane and tissue sheaths could be stained by a silver impregnation method; they ar described in the gut of grasshoppers by Riedel (1946) and in blowfly larvae b Waterhouse (1950). But none of these authors considers the formation of thi tissue. The only detailed work on that subject is by Lazarenko (1925) wh used supravital methylene blue to show how widespread these thin sheets of connective tissue are in the larva of *Oryctes*. Lazarenko also implanted tubes of celloidin into the larvae and observed that these objects became enclosed in capsules formed by spindle-shaped cells which later fused to a syncytium in which connective tissue fibres appeared. From these observations he inferred that the normal connective tissues of insects are probably formed in the same way. The cells in question he regarded not as blood-cells but as special mesenchyme cells from the tissue spaces. An alternative view is that the tracheal cells unite with one another to form a fenestrated membrane which constitutes the 'peritoneal membranes' and indefinite connective tissues of insects (de Sinéty, 1901; Riede, 1912; Remy, 1925; Dreher, 1936). Membranes of this type may well exist, but they certainly do not include the basement membrane in *Rhodnius* (Wigglesworth, 1954) nor the other membranes considered in this paper.

The encapsulation of foreign bodies by blood-cells in insects has been described by many authors. Clumps of tubercle bacilli are encapsulated by conglomerations of leucocytes in *Galleria* (Metalnikov, 1908); but according to Hollande (1930), in this cyst formation there is nothing comparable with the connective tissue formed by the fibroblasts of vertebrates. Similar observations were made by Iwasaki (1927), Boese (1936), Rooseboom (1937), and others after the introduction of Indian ink, parasites, &c. Lartschenko (1933), who describes the encapsulation of the eggs of parasites in the larva of *Pieris*, accepts the view of Lazarenko. But Ermin (1939), who gives a useful survey of the literature on the blood-cells of insects, again emphasizes that the conglomerated sheets of blood-cells which form in *Periplaneta* around injected Indian ink or around dead yeast-cells, are not really comparable with the connective tissue of vertebrates.

The results reported in the present paper agree with these conclusions. Fig. 5 shows a small part of the capsule formed around blood-cells laden with Indian ink. It is clear that these cells are not only forming a cellular sheath; they are giving rise to free membranes of 'mucopolysaccharide'. But these membranes are very fragile structures, easily dispersed, and not to be compared with fibrous tissue. Precisely the same mechanism of formation is seen in the normal connective tissue around and between the muscle fibres.

The cells which take part in this process are the commonest type of haemocytes; they are here called amoebocytes. Whether they are free in the blood or adherent to the tissues, they are always recognizable by their 'mucopolysaccharide' contents. It seems unnecessary to separate them as connective tissue-cells. Indeed, these cells in *Rhodnius* are now recognized as having at least three functions: they are the most actively phagocytic of the haemocytes; in the early stages of moulting they seem to play some essential part in the production of the moulting hormone by the thoracic glands; and in the later stages of moulting they liberate the 'mucopolysaccharide' material which forms or contributes to the connective tissues and basement membranes.

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The Fine Structure of the Scales and Teeth of *Raia clavata*

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With one plate (fig. 1)

SUMMARY

The fine structure of the scales of *Raia clavata* has been determined by means of the polarizing microscope and X-ray analysis. Each scale consists of a cone of dentine covered by an enamel-like layer and both of these tissues contain crystallites of the mineral hydroxyapatite. In each case the crystallite size is similar to that of mammalian dentine possessing the same mineral. However, whereas the dentine of *Raia* contains both collagen fibres and mineral crystallites arranged parallel with the surface of the scale, the enamel-like layer has a low organic content and its crystallites lie with their optic axes at right angles to the surface. These differences in crystallite arrangement and organic content result in different optical properties in the two tissues. Nevertheless, both tissues are produced by the mesoderm cells of the dentine papilla, and in this respect the enamel-like tissue differs fundamentally from the ectodermal enamel of mammalian teeth.

INTRODUCTION

KEIL (1934, 1935, 1936, 1938, 1939), Schmidt (1924, 1934, 1937), and Thewlis (1940) in particular have studied the minute structure of teeth. The results of their work may be summarized as follows:

(*a*) In both dentine and enamel the chief mineral constituent is hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$.

(*b*) Enamel mineral consists of crystallites with an average size of about 0·3 μ. These are arranged with their optic axes in the same general direction as the enamel prisms, although deviation from the prism axis may be up to 40°.

(*c*) The average size of the crystallites in dentine is about a tenth of that in enamel. In human dentine the orientation of crystallites is 'spheritic', although in the teeth of lower vertebrates the mineral crystallites are orientated in a similar direction to the collagen fibres.

(*d*) Enamel has a low organic content; its optical properties in polarized light are due entirely to mineral. However, dentine is composed of about 70% mineral and 30% organic material and water. The optical properties of intact dentine are due mainly to collagen fibres, which are sufficiently optically active to over-compensate the mineral crystallites. Only when the collagen is removed (e.g. by glycerol-potash treatment) can the mineral properties be determined.

The results discussed above were gained from the examination of mammalian, and in particular human teeth. Only a few similar investigations (Schmidt,

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1938, 1940, 1948) have been made of the tooth structure of lower vertebrates which will be one of the objects of the work detailed here.

Besides differing in submicroscopic structure, mammalian enamel and dentine may be distinguished by more obvious structural characters. Enamel i produced by the outward growth of the epithelial ameloblast cells of th enamel organ, each ameloblast forming an enamel prism; it is, therefore *ectodermal* in origin and prismatic in structure. On the other hand, dentine i formed by the inward growth of *mesodermal* odontoblast cells and is of a tubular nature. Furthermore, the organic matrix of dentine consists mainly o collagen fibres, whilst that of enamel is keratinous.

Early research on the tooth structure of lower vertebrates, particularly fish revealed that the relationship between the different tissues was by no means so clear, and considerable controversy arose. The enamel-like layer covering teeth and scales is never prismatic and frequently is not clearly marked off from the dentine below; furthermore, investigations into the origin of the enamel-like tissue led to different conclusions. Some workers (e.g. Applebaum, 1942; Lison, 1946) concluded that this layer was of ectodermal origin, while others (e.g. Tomes, 1898; Bargmann, 1937) believed it to be a mesodermal product. The suggestion was also made that this tissue had a mixed origin (Thomasset, 1926).

However, as a result of more complete investigations a better understanding of the problem now exists. After careful examination with the polarizing microscope, Schmidt (1938, 1940, 1948) came to believe that the enamel-like layer of the teeth of cyprinoids and certain sharks (*Oxyrhina* and *Carcharias*) was fundamentally the same as the dentine supporting it. The reasons for this were:

(*a*) Developing mammalian enamel grows outwardly from the amelodentinal junction, whereas the enamel-like tissue of the teeth under consideration appeared to extend inwardly.

(*b*) Mammalian enamel and dentine are both positively birefringent with respect to the surface of the tooth. This is because the negatively birefringent hydroxyapatite crystallites in the enamel lie with their optic axes at *right angles* to the surface, whilst the positive collagen fibres of the dentine run *parallel* with this direction. Normally the mineral crystallites in the dentine are parallel with the collagen but sometimes, as in mammalian dentine, a spheritic orientation of crystallites may exist. In either case the birefringence of the collagen fibres predominates.

Mineral crystallites in the fish teeth examined were all found to be arranged with their optic axes *parallel* with the surface of the tooth. In the deeper-lying dentine the birefringence of the collagen predominated as in mammalian dentine; outside this was a zone where the collagen and mineral just compensated each other, producing anomalous polarization colours, and in the outermost tooth layer the mineral predominated. This means that the sign of birefringence of the outer layer is opposite to that of the deeper-lying dentine; quite unlike the condition in mammalian teeth. This seems to have come about by the resorption of the collagen from the outermost layer of dentine, and the hypercalcification of this layer. Schmidt suggests the term 'Durodentin' to describe this layer and distinguish it from mammalian enamel.

Mention must also be made of the work of Levi (1939, 1940*a*) and Kvam (1946, 1950) on the development of teeth. Between them, these workers have covered a wide range of fish species and although an adequate summary of their results cannot be given here, it may be said that in every case the enamel-like tissue was formed as a result of the calcification of a collagen matrix produced by the odontoblast cells of the dentine papilla. This tissue is therefore a *mesodermal* product. Further reference to these results will be made later.

It is hoped that the following account of the teeth and scales of *Raia clavata* will illustrate how the fine structure of such tissues may be determined, and how the results of such determinations agree with the conclusions of the workers mentioned above.

MATERIAL AND METHODS

Specimens of the thorn-backed ray (*R. clavata*) were obtained from the Marine Laboratory at Plymouth; they were fixed in either Bouin, neutral formalin, or alcohol as they were caught.

Sections of the scales were prepared simply by grinding on frosted glass with varying grades of carborundum powder. Smaller scales were embedded in Canada balsam, which was allowed to dry before grinding. All sections were polished by rubbing in water first on frosted glass, and then finally on plain glass.

It was found necessary to investigate the development of the teeth. Since the jaw material hardened considerably and could be sectioned only with difficulty when embedded in paraffin, a celloidin method was used (the low viscosity nitrocellulose technique of Chesterman and Leach (1949)).

The teeth develop continuously under a fold of the oral epithelium immediately behind each jaw. Thus a section across the jaw will yield a series of teeth at progressive stages of development (see also Tomes, 1898). The cartilage of the jaw was dissected away before embedding in order to facilitate the eventual section-cutting.

X-ray investigations were carried out in the Physics and Geology Departments of the University of Bristol, mainly with copper targets (see Thewlis, 1940). Attempts were made to compare the patterns of different calcified tissues and to determine crystallite orientation.

THE STRUCTURE OF THE SCALES

A vertical section of the scale of *R. clavata* shows an enamel-like tissue covering dentine. The dentine is rather coarse-tubed and is separated from the enamel by a well defined border region.

Detailed microscopic examination reveals that the outer enamel-like layer is penetrated for varying distances by processes arising from the border region. Unlike the condition in the teeth and scales of other elasmobranchs these processes are not continuations of the dentine tubules, for the latte actually terminate some distance below the border region. The processes in the outer enamel-like layer appear to be fibrous rather than tubular.

Apart from these penetrating processes, the enamel-like layer possesses little or no structure, although it is hard, brittle, and transparent. Frequently it breaks away from the dentine during sectioning; the line of fracture is always along the border region, the dentine remaining intact.

In polarized light (fig. 1, A) the outer layer shows up brightly, the sign of birefringence being positive with respect to the surface of the scale. The junction region shows very little activity, being slightly negative, whilst the dentine is positively birefringent like the outer layer. Treating a section with liquids of varying refractive index has no effect on the enamel-like layer and decalcification leaves only a slight organic residue. This low organic content and absence of 'form' birefringence indicates that the optical activity of the enamel-like layer is due entirely to mineral.

The dentine showed a slight increase in activity when decalcified, although the sign of birefringence was unaltered. Thus it must be deduced that as in teeth of higher forms, the collagen fibres, which are positively birefringent with respect to their length, must be parallel with the surface and mainly responsible for the optical properties of the dentine.

If the collagen is removed from the intact dentine by boiling in 10% glycerol-potash solution, the optical properties of the mineral are seen. The extracted dentine becomes negatively birefringent with respect to the surface provided that it is allowed to imbibe a liquid possessing a refractive index (e.g. aniline, R.I. 1·58) sufficiently high to remove 'form' birefringence. In such an extracted section the enamel-like layer retains all its original properties but the junctional zone disappears, with the result that the dentine becomes separated from the enamel-like layer.

The results so far may be summarized thus. The enamel-like layer is markedly birefringent and its properties are entirely determined by mineral. On the other hand, the optical properties of the dentine are determined mainly by collagen, which, in intact dentine, overcompensates the mineral, which possesses an opposing birefringence.

A further analysis of the mineral was made by means of X-rays. The 'powder method' used for the identification of minerals shows that the patterns produced by mammalian enamel and dentine, whilst not identical, both resemble

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FIG. 1 (plate). A, part of a vertical section passing through the tip of a scale of *Raia clavata* as it appears in polarized light. The enamel-like layer shows up brightly and it is penetrated in places by processes arising at its innermost edge. Below this is the tubular dentine, the outer layer of which is hypercalcified and therefore appears darker than the rest of the dentine.

B, X-ray orientation pattern of the edge of a scale, showing four arcs.

C, edge of the scale with the enamel-like layer removed, showing only two arcs.

D, vertical section through an early tooth germ of *Raia clavata* stained with haematoxylin and eosin. The outer layer consists of a packed mass of fibres which arise from the odontoblasts. This eventually becomes the enamel-like layer of the adult tooth.

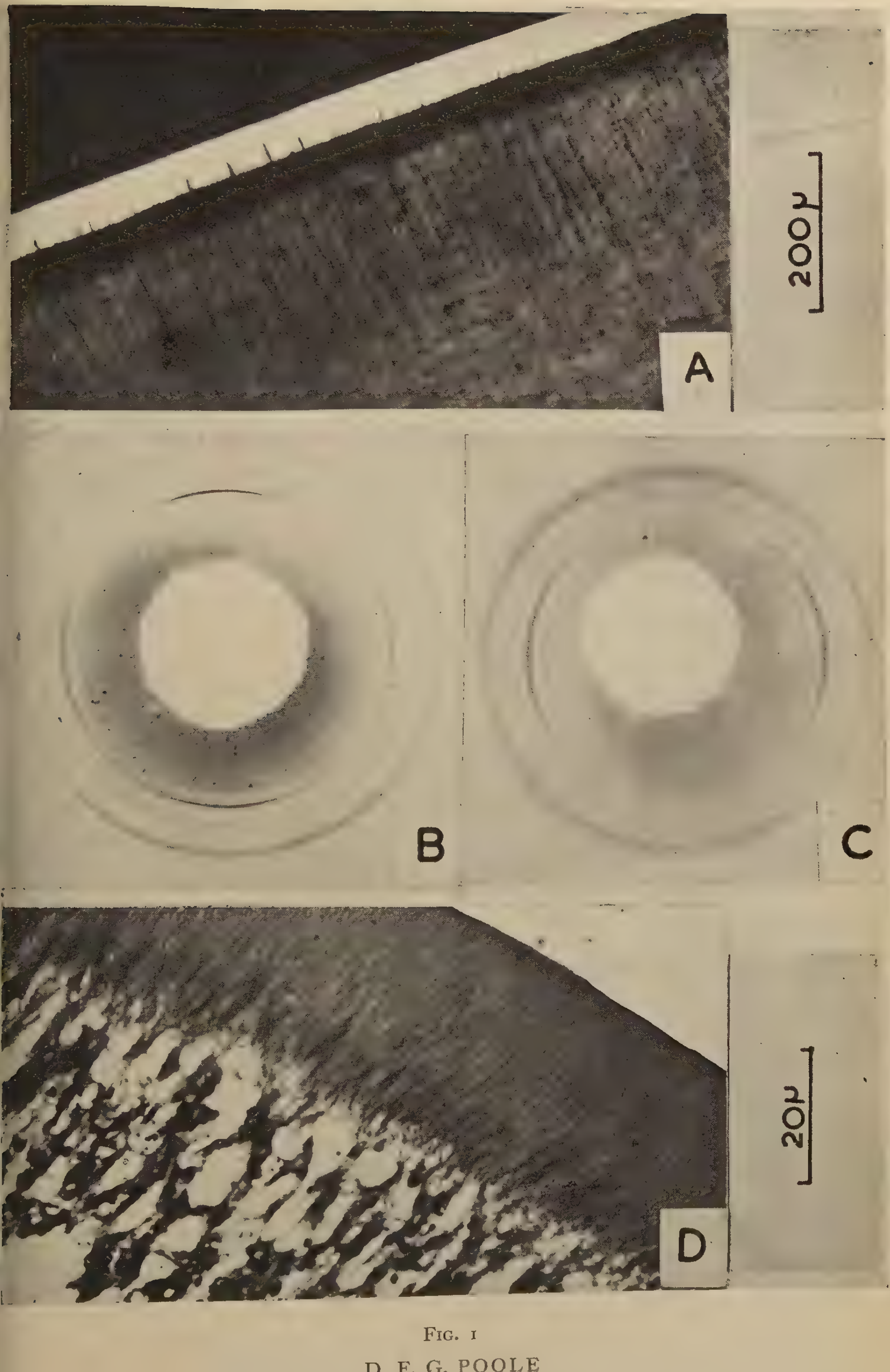

Fig. 1

D. F. G. POOLE

the pattern produced by the mineral hydroxyapatite (Thewlis, 1940). It is generally believed that the difference in the X-ray patterns of enamel and dentine is due to the difference in the size of the crystallites in the two tissues.

However, patterns produced by the mineral of the enamel-like layer and of the dentine of *R. clavata* proved to be identical and both were very similar to that given by mammalian dentine. Therefore, it is to be concluded that the mineral of the enamel-like layer and the dentine is the same in chemical nature and crystallite size. The similarity to mammalian dentine suggests that ray mineral is hydroxyapatite, with a crystallite size of about 0·03 μ.

Crystallite orientation was also determined by an X-ray diffraction technique in the same way as the orientation in human teeth has been determined (Thewlis, 1940). If a section of mineral is placed at right angles to a beam of X-rays, the diffraction pattern produced by the mineral may be recorded on a sheet of film placed behind the section. When the crystallites in such a section are arranged at random a pattern of concentric circles is produced, but if any preferential orientation of the crystallites exists, the circles of diffraction are reduced to arcs corresponding to the direction of orientation. In this way it was found that the mineral crystallites of human enamel are orientated approximately parallel with the axis of the prism.

Unfortunately, the enamel-like layer of the scales under consideration is so thin that it was impossible to mount a section in such a way that only the outer layer was struck by the beam of X-rays. As a result it was necessary to record the pattern produced by the beam striking the edge of the scale—a combination of both the enamel-like layer and the dentine—and then to grind away the outer layer and record the pattern produced by the dentine alone. The two photographs were then compared; any diffraction lines present in the first but absent in the second photograph must have been produced by the outer enamel-like layer.

The patterns produced in the two cases may be seen in fig. 1, B and C. The first pattern shows two vertical and two horizontal arcs, but the second pattern shows only the two horizontal arcs. Since the second pattern is produced by dentine alone, the two vertical arcs in the first pattern must have been produced by the enamel-like layer.

The section was mounted so that the surface of the scale was horizontal, and therefore the production of vertical arcs by the enamel-like layer indicates that the mineral crystallites must be arranged at right angles to the surface of the scale. Similarly, the horizontal arcs produced by the dentine indicate that the crystallites here must be arranged parallel with the surface. However, the horizontal arcs are greater than the vertical, so that the orientation in the dentine is not so perfect as in the enamel-like layer. The deeper-lying dentine had a similar orientation but much weaker lines, suggesting that the degree of calcification is lower towards the pulp cavity.

X-ray analysis therefore indicates that all the mineral in these ray scales is similar to hydroxyapatite and that it consists of crystallites of the same size as those of mammalian dentine. In the outer enamel-like layer there is a well marked orientation of crystallites at right angles to the surface, but in th dentine there is less well defined orientation parallel with the surface of th scale (see fig. 2).

It is now possible to understand the optical properties of these tissues mor clearly. Hydroxyapatite possesses an intrinsic negative birefringence, but th arrangement of crystallites with their optic axes at right angles to the surface

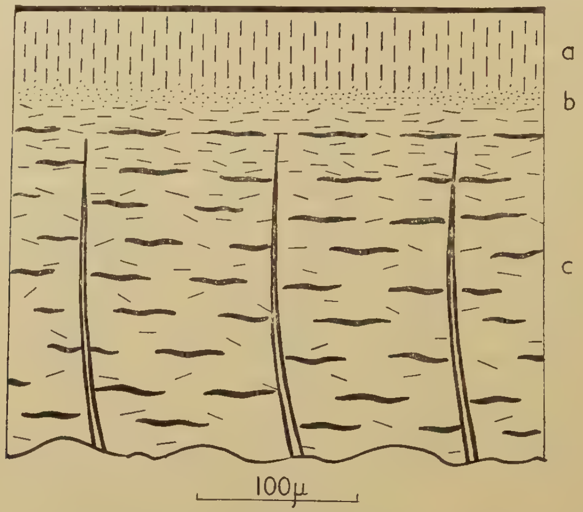

FIG. 2. The fine structure of a section of *Raia* scale shown diagrammatically. *a*, outer enamel-like layer containing mineral only; the crystallite axes are perpendicular to the surface. *b*, inner uncalcified region of the enamel-like layer. *c*, dentine with both collagen fibres and mineral crystallites, the latter being more numerous towards the outside. In addition the dentine is penetrated by tubules which originate at the pulp cavity.

Note: the crystallite and optic axes of apatite are coincident.

as in the outer layer of the ray scale, gives this layer a positive birefringence with respect to the surface. An arrangement of similar crystallites parallel with the surface would give a negative birefringence with respect to the surface— exactly the condition found in extracted dentine.

In polarized light the outermost dentine is slightly less refractive than the deeper-lying dentine (fig. 1, A). X-ray analysis shows the outer dentine to possess a rather higher degree of calcification than that nearer the pulp cavity. Since the collagen fibres and mineral crystallites are approximately parallel, it is evident that the slight hypercalcification of the outer dentine results in the negative crystallites compensating the positive collagen to a greater extent than in the deeper lying dentine. The total activity of this layer is therefore reduced.

THE FORMATION OF THE CALCIFIED TISSUES

The teeth of *R. clavata*, although small and flattened, possess a structure identical with that of the scales. Since their development is easily studied, it was possible to follow the formation of the calcified tissues already described.

The beginning of tooth formation is marked by an aggregation of mesoderm cells under a fold of the oral epithelium immediately behind each jaw. No complex enamel organ is produced, and the mesoderm cells stain readily with haematoxylin. Soon afterwards a fibrous organic matrix differentiates under the epithelium; these fibres were most clearly seen in material which had been fixed in alcohol to preserve any mineral salts present, although the general histological picture of such preparations was poor (fig. 1, D). The fibres had no well defined staining properties, but it was possible to observe that they possessed a positive birefringence with respect to their length. This birefringence was reversed when a section was treated with aniline or aqueous phenol solutions, suggesting that the fibres have a collagenous nature (Frey-Wyssling, 1948).

Calcification of this matrix results in the enamel-like layer covering the teeth and scales; as calcification proceeds the fibres are resorbed. The mineral crystallites lie with their optic axes at right angles to the surface of the tooth, and since the collagenous fibres also run in this direction it is possible that they are responsible for the orientation of the crystallites. Resorption and calcification are incomplete along the innermost border of the enamel-like layer and the uncalcified inner ends of the fibres remain even in adult teeth.

The dentine development was similar to that of mammalian dentine. A layer of predentine is formed first; this possesses dentine tubules and stains only slightly with haematoxylin. After calcification this matrix stains heavily, showing the globular outlines of the calcospherites. Dentine formation does not appear to begin until after the calcification of the outer layer has already begun.

DISCUSSION

The enamel-like layer of the *Raia* scale possesses optical characteristics which make it quite distinct from the dentine supporting it. As shown by Schmidt, this is not the case in sharks such as *Oxyrhina* and *Carcharias*, where the optical properties of the teeth revealed that the enamel-like layer was normal dentine which had been hypercalcified. To a certain extent, the crystallite arrangement in the 'enamel' of *Raia* resembles that in the mammalian enamel, but here the similarity ends. Apart from the absence of prisms, *Raia* 'enamel' differs in several other important respects.

X-ray 'powder' analysis of mammalian teeth shows that the patterns given by enamel and dentine are not the same, probably because of the difference in size of the mineral crystallites in the two tissues. It has been seen here that the patterns given by the tissues of the *Raia* scale are identical, and that both are similar to the pattern of mammalian dentine. Thus all the mineral in the *Raia* scale must be the same as that in mammalian dentine.

The crystallite orientation in the enamel-like layer of the ray scale is probably to be attributed to the nature of the organic matrix in which the mineral was deposited. The latter consisted initially of a packed mass of fibres with certain collagenous properties; these fibres run outwards and at right angles to the surface of the tooth. As mineral is deposited the fibres are resorbed an the crystallites come to lie parallel with the original fibre direction. Resorptic is incomplete, the inner ends of the fibres remaining uncalcified and producin the appearance of a junction between the enamel-like layer and the dentin It may be recalled that in adult scales this zone possesses a low degree birefringence, is a line of weakness along which fractures readily occur, an disappears when a section is extracted with glycerol-potash solution. The properties are to be expected of a region composed of collagen fibres calcifie to only a slight extent.

One of the most important points concerning tooth formation in *Raia* is th formation of the organic matrix by the odontoblast cells of the dentine papill There is no evidence of the ameloblasts participating in the production organic material and this finding is in complete agreement with the results Levi and Kvam. In other words, the enamel-like layer is of *mesodermal* origin being formed as the first differentiated portion of the dentine papilla. It is interest to note here that in mammals, a layer of dentine is already presen *before* the formation of *ectodermal* enamel begins.

The condition in the teeth and scales of the ray, where the enamel-like laye possesses clearly defined optical properties, is not found in all fish. In additio to the sharks described by Schmidt, the writer has also found in the teeth an scales of fossil sharks (*Otodus* spp. and others) and of the skate (*Raia bati* that the enamel-like layer has poorly defined optical properties. In teleosts the 'enamel' tends to be restricted to the tip of a tooth and this 'enamel' con is highly calcified in the teeth of both the hake (*Merluccius vulgaris*) and th cod (*Gadus morrhua*). Despite this, the degree of birefringence is much lowe than that of the dentine below, suggesting that the mineral crystallites have poorly ordered arrangement, tending to cancel out the optical effects of each other. Kvam showed in *Gadus virens* that the organic matrix of the enamel-like tip consists of collagen fibres whose general direction is longitudinal bu nevertheless have a twisting path. If such fibres orientate the mineral crystallites, it is to be expected that the resulting tissue will have anomalous optica properties.

Examination of the teeth of many species of fish in this way reveals tha there is considerable variation in the form and structure of the enamel-lik layer. Yet, as shown by Levi and Kvam, there is one common feature throughout and that is the formation of the enamel-like layer as the first differentiate portion of the dentine papilla. Because of its function and origin, Kvam suggests the term 'mesodermal enamel' for use in describing the outer layer o fish teeth and scales. In view of the variation in structure of this tissue in different species, this general term of description is probably the most suitable.

One final point concerns the function of the epithelial cells covering the developing tooth germs. Most descriptions of tooth formation in fish make note of the considerable changes in size and shape of these cells, as well as changes in the position of the nuclei which occur as tooth formation proceeds.

There is no evidence that they contribute to the organic matrix of developing teeth; so what is their function?

It is still possible that they furnish mineral salts for the calcification of the outer tooth layer, although in *Raia*, observations with the polarizing microscope suggest that calcification of this layer begins at the outside and proceeds inwards. This would mean that the mineral salts here are being provided probably by the cells of the dentine papilla. Nevertheless, there is evidence to the contrary that in certain sharks the direction of calcification is outwards (Applebaum, 1942). More evidence on this point would be useful, particularly as to the presence or absence of alkaline phosphatases in these epithelial cells.

In the opinion of Schmidt (1940) the epithelial enamel organ in fish is responsible for the shaping of the tooth, and the possibility must also be considered that these cells may have something to do with the organization of the dentine. In mammals it appears that the ameloblast cells organize the transformation of mesenchyme cells into odontoblasts and cause them to begin dentine formation; there seems to be no reason why this should not also be the case in fishes. However, these minor points are speculative and are presented somewhat in the hope of stimulating further research into the subject.

In conclusion, the author wishes to express his thanks to Professor J. E. Harris of the Zoology Department and Professor A. I. Darling of the Dental School, University of Bristol, for their instigation and continued encouragement of all the work carried out. Thanks are also due to the various members of the Physics and Geology Departments and Dental School of the University for help received.

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The Thickness of the Cortex of the Sea-Urchin Egg and the Problem of the Vitelline Membrane

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With one plate (fig. 1)

SUMMARY

The gelated cortex of a sea-urchin egg can be seen as a granular layer at the edge of the hyaline zone in a centrifuged egg. Measurements were made of the thickness of this layer in the eggs of *Paracentrotus lividus* and *Arbacia lixula* at various stages of development from the unfertilized egg to the first cleavage. The thickness was roughly 2 μ in living eggs, and 1·15–1·35 μ in sections of fixed eggs. There were no appreciable changes in the thickness up to the first cleavage, and it is concluded that a value of 1·5 μ can be taken as an approximate figure for all stages.

The cortex is usually invisible in normal eggs either living or in section, but in the case of sections of fertilized *Arbacia* eggs it can be seen as a vacuolated layer. The thickness of this layer was found to be 1·13 μ at the sperm aster stage and 1·50 μ at cleavage. In these eggs at cleavage, there were no signs either of differences in thickness at different regions of the surface or of a differentiated region of the cytoplasm ahead of the furrow.

There were no clear indications of the presence of a vitelline membrane either in living or fixed eggs.

A layer about 0·8 μ thick, pale with iron haematoxylin, was found at the edge of sections of unfertilized eggs which had been fixed in Bouin, but not with those which had been fixed in Flemming. This is probably the outer layer of the cortex which normally contains the cortical granules.

INTRODUCTION

THE work described in this paper was mainly done in order to get a reliable measure of the thickness of cortex of the sea-urchin egg. This figure is essential for the interpretation of the measurements of the mechanical properties of the cell surface which are given in a series of papers by Mitchison and Swann (1954*a*, 1954*b*, 1955). It is also of some general interest in being a piece of important information about the cell membrane in what is perhaps the most widely-studied single cell in the animal kingdom.

The cortex, first described by Chambers (1917), is a gelated layer, a micron or two in thickness, at the surface of the egg. It is presumably responsible for most of the mechanical properties of the surface (e.g. elasticity, expansive power) and can be regarded as the 'structural layer' of the cell membrane (Mitchison, 1952). On the outside of the cortex there is probably a very thin 'permeability layer' or plasma membrane, mainly lipid in nature, which is invisible but whose presence can be inferred from the permeability and electrical properties of the cell membrane. These two layers make up the basic cell membrane which exists at all stages of development and which cannot be

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separated from the rest of the cell without causing cytolysis. There are also least four other surface layers outside the cell membrane which exist on during certain stages of development: the 'vitelline membrane' (whose exi tence will be discussed later) and the jelly in the unfertilized egg; and t hyaline layer and the fertilization membrane in the fertilized egg. Recentl Hiramoto (1954, 1955) has shown that there is another layer in fertilized eg lying outside the hyaline layer.

The cortex is a difficult structure to detect or measure. It does not diff from the egg cytoplasm in light absorption or in refractive index, so it is impossible to see it directly in the living cell either by ordinary or phase-contra microscopy. It is also impossible to stain it in a section, though it can be see in the sections of some eggs at some stages of development because it contai granules different from those in the cytoplasm. The only detectable ways which the main material of the cortex differs from the cytoplasm are in i gelation or mechanical properties, in its birefringence, and in its capacity scatter light; and it is therefore necessary to use one or other of these properti for measuring the cortical thickness. The easiest reliable method seems to b to use eggs which have been stratified in a centrifuge. At the centripetal en the gelated cortex, which has granules stuck in it, can be seen as a layer sepa ate from the clear fluid cytoplasm whose granules have been forced down t the other end of the egg.

Most of the work to be described consists of measurements on the cortic thickness in centrifuged eggs, both living and in stained section; but som other observations were also made on normal uncentrifuged eggs in order t discover what surface layers were visible under the best optical condition and, in particular, to find whether a vitelline membrane could be seen at th surface of unfertilized eggs.

MEASUREMENTS ON CENTRIFUGED EGGS

Living eggs

In this and subsequent experiments the two species of sea urchin used wer *Paracentrotus lividus* and *Arbacia lixula*. The eggs were placed in centrifug tubes which contained a layer of sea-water at the top and at the bottom a laye

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FIG. 1 (plate). Eggs of *Paracentrotus lividus* and *Arbacia lixula*.

A, *Paracentrotus*, unfertilized, living, centrifuged. B, *Arbacia*, unfertilized, living, centrifuge C and D, *Arbacia*, unfertilized, living, centrifuged, dark-ground illumination. E, *Paracentrotu* unfertilized, centrifuged, fixed in Flemming, section stained with iron haematoxylin (I.H. F, *Arbacia*, unfertilized, centrifuged, fixed in Flemming, section stained with I.H. G and *Paracentrotus*, unfertilized, living. I, *Paracentrotus*, unfertilized, living, treated with trypsi J, *Paracentrotus*, sperm aster stage, living. K, *Paracentrotus*, unfertilized, fixed in Bouin, sectio stained with I.H. L, *Arbacia*, unfertilized, fixed in Bouin, section stained with I.H. M, *Paracen trotus*, unfertilized, fixed in Flemming, section stained with I.H. N, *Paracentrotus*, sperm ast stage, fixed in Bouin, section stained with I.H. O, *Arbacia*, early sperm aster stage, fixed Flemming, section stained with I.H. P, *Arbacia*, cleavage, fixed in Flemming, section staine in I.H.

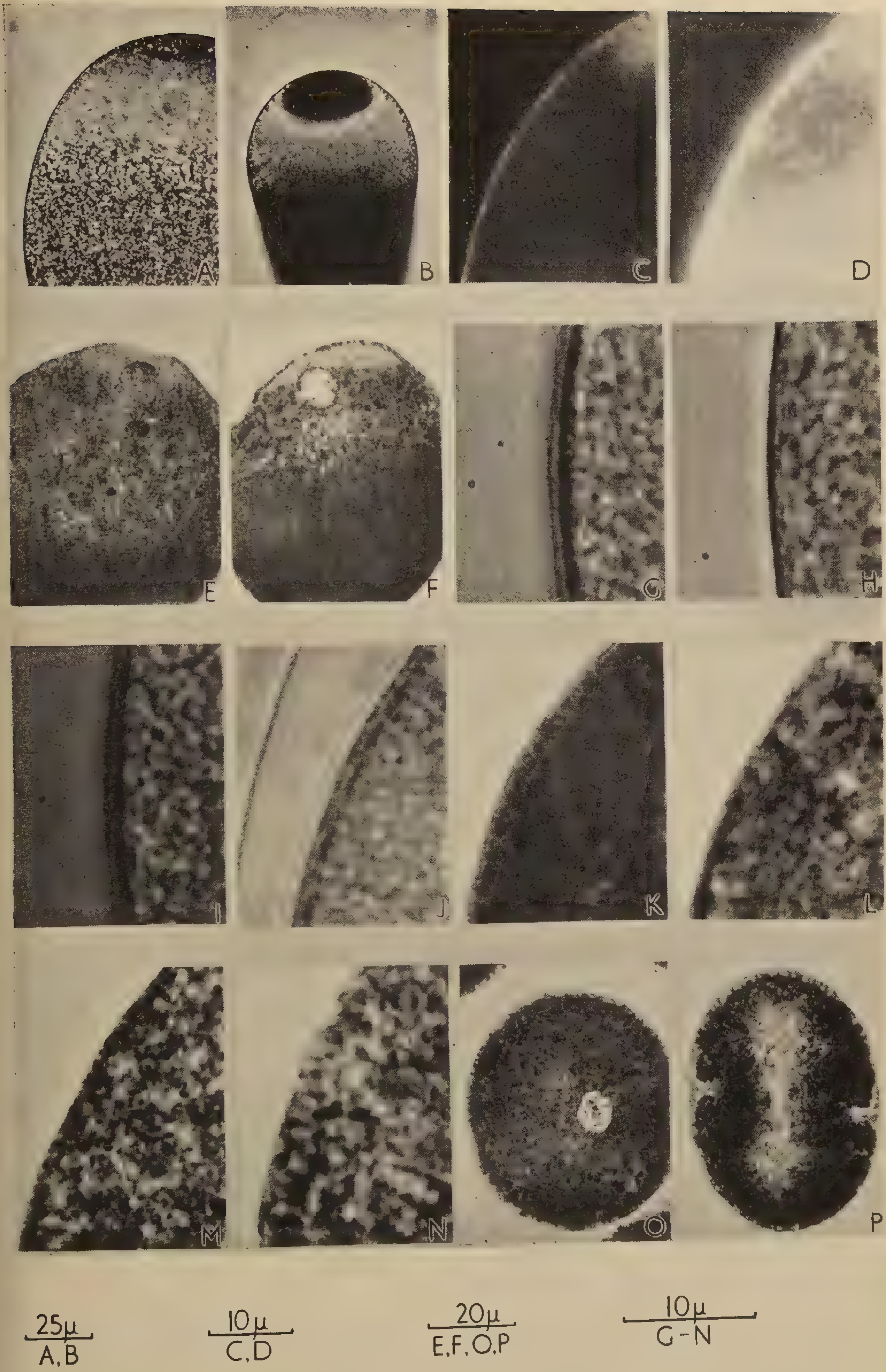

FIG. 1

J. M. MITCHISON

f 1·1 M sucrose, which was isotonic with sea-water but denser than the eggs. slight stirring at the boundary between these two layers produced a density radient in which the eggs came to rest. The eggs remained in the gradient uring centrifuging and so avoided the damage which would have been caused y flattening against the bottom of the tube if they had been centrifuged in ea-water. They were centrifuged in this gradient with forces up to 18,000 g, emoved and compressed under a coverslip (to about 20μ thickness), and then xamined with a 4-mm dry apochromatic objective (N.A. 0·95) and a micrometer ocular.

The *Paracentrotus* eggs were difficult to stratify, and 5–10 minutes' centriuging at 18,000 g was needed to give a sharp separation of the components. he eggs stratified with an oil cap at the centripetal end, and with the yolk ranules and mitochondria at the centrifugal end. In between there was a zone f more or less clear cytoplasm which contained the nucleus, and at the edge f this zone the cortex could be seen as a thin granular layer (fig. 1, A). The ggs were centrifuged at three stages—unfertilized, sperm aster, and early leavage—but they behaved in substantially the same way in each case and the resence of asters in the last two stages had comparatively little effect on the tratification.

From measurements on 10–20 eggs, the thickness of the granular cortical ayer was about 2μ. It was more difficult to measure this layer in the thick iving eggs than it was in sections, and the accuracy of the measurements was ot greater than ±0·5μ. Within these limits (1·5–2·5μ), there was no differnce in the cortical thickness at the three stages. There was also no difference n the thickness at different speeds of centrifuging. If the eggs were centriuged at a lower speed or for a shorter time, the clear layer was thinner and ess sharply defined but the cortex was equally thick.

The *Arbacia* eggs were easier to stratify and only needed 10 minutes' centriuging at 10,000 g. The pigment granules also were larger, and, especially in he fertilized eggs, the cortex was clearly defined by a single layer of these ranules. Apart from this, the *Arbacia* eggs gave similar results to the *Paraentrotus* eggs. The cortex was about 2μ thick (±0·5μ) in fertilized, sperm ster, and early cleavage stages, and there was no detectable change in thickess at different speeds or times of centrifuging. A centrifuged unfertilized gg is shown in fig. 1, B.

Lindahl (1932) examined centrifuged sea-urchin eggs (*Arbacia* and other pecies) under dark-ground illumination and found two luminous layers at he edge. He discussed in some detail the interpretation of this appearance in erms of an arrangement of membranes, and, in view of this, it seemed worth epeating his observations. When examined with a high power dark-ground ondenser (Zeiss Siedentopf condenser and apochromatic oil-immersion -mm objective, N.A. 0·6), a centrifuged unfertilized *Arbacia* egg showed a attern at the edge with the two luminous layers, provided the light intensity vas low (fig. 1, C). The thick inner one was evidently the cortex and, about 1μ utside it, there was a much thinner layer. When, however, the light intensity was raised, a number of other layers appeared on the outside. This was d both to the increase in total light intensity of the pattern and also to the i creased contrast sensitivity of the eye with the higher general level of illumin tion. This latter effect has been simulated in fig. 1, D, which shows what t edge of the egg looked like with a high light intensity. This print, however, made from the *same* negative as was used for fig. 1, C, but with bromide pap of much higher contrast. It can be seen from these photographs that althou the inner of Lindahl's two layers represents a real structure (presumably t cortex), it is more than likely that the outer layer is simply the first of the set diffraction bands caused by the light scattering of the cortex. This is not ce tain, owing to the lack of an adequate optical theory, but it seems very pro able in view of the equal spacing of all the rings outside the cortex.

Sections

Measurements were also made of the thickness of the cortex in sections fixed eggs. This method increases the accuracy of the measurements becau better optical arrangements are possible than with living material, but it h the disadvantage of introducing shrinkage during the fixation.

Eggs of *Paracentrotus* and *Arbacia* were centrifuged at three stages of d velopment as before, fixed in Flemming or Bouin, cut at 7μ, and stained wi Heidenhain's iron haematoxylin. The sections were then examined under th best optical conditions (Zeiss 3-mm apochromatic oil-immersion objectiv N.A. 1·4, achromatic condenser, and green light). The stratification is le marked than in the living eggs, and the cortex, though visible at the edge of th clear layer, is most clearly defined at the centripetal end (fig. 1, E and F). In th living egg this end is obscured by the oil layer, but during fixation and stainin the oil is dissolved away. The thickness of the cortex was measured on 25 egg at each stage by means of a filar micrometer eyepiece mounted separatel above the microscope to avoid vibration. These results had to be corrected fo the shrinkage during fixation. The best way (though it is somewhat arbitrary seemed to be to assume that the cortex had shrunk by the same proportion a the overall size of the eggs. Accordingly the maximum and minimum diamete of 25 of the largest sections was measured at each stage, and compared wit the average of fresh eggs. The average cortical thickness (and the standar deviation) was then multiplied by the factor—fresh egg diameter / fixed eg diameter—appropriate for that stage. The average factor for the three stage in Flemming was 1·18 for *Paracentrotus* and 1·41 for *Arbacia*. The correcte results for the thickness of the cortex in eggs fixed in Flemming are given i table 1. Eggs fixed in Flemming were used for most of the measurements sinc the cortex was rather more sharply defined than in Bouin, but comparativ measurements showed no significant differences in the cortical thicknes between eggs at the same stage in the two fixatives. The layer, pale with iro haematoxylin (see p. 114), which occurred at the edge of the eggs fixed i Bouin, was included in the measurements of cortical thickness, but it was les clearly defined in these centrifuged eggs than in the uncentrifuged ones.

TABLE I

Thickness of the cortex in centrifuged eggs fixed in Flemming
(Corrected for shrinkage)

Species	*Stage*	*Average thickness in μ from 25 eggs*	*Standard deviation in μ*
Paracentrotus lividus	unfertilized	1·15	0·21
	sperm aster	1·20	0·24
	early cleavage	1·13	0·23
Arbacia lixula	unfertilized	1·20	0·26
	sperm aster	1·35	0·35
	early cleavage	1·17	0·31

OBSERVATIONS ON NORMAL EGGS

Living eggs

In the course of making observations on the cortex in centrifuged eggs, it lso seemed worth while to examine the edge of normal eggs, living and in ections, and to see in general what layers were visible under good optical onditions, and in particular whether a vitelline membrane could be seen at he surface of unfertilized eggs. The living eggs were compressed under a overslip and examined with a Zeiss 2-mm water-immersion achromat, N.A. ·18, used with green light. This lens is designed to work through a coverslip nd probably gives the best resolution that can be obtained on a preparation n water and under a coverslip. In theory it should give slightly better results han an oil-immersion lens on such a preparation, and this was borne out in ractice.

When examined under these conditions, the edge of an unfertilized *Para-entrotus* egg showed a series of dark rings diminishing in thickness and in ontrast from the inside outwards (fig. 1, G). The first impression of the two nnermost rings was that they defined a thin membrane on the surface of the gg. This point will be discussed later, but we can anticipate the discussion by aying that no definite conclusions can be drawn about the presence of such a nembrane from the appearance of these rings. The spacing from peak to peak f these two rings was about 0·8μ. There was no doubt that the rings further ut were diffraction bands, with a spacing of about 0·6μ.

The pattern at the edge of the egg depended very critically on the focus, and slight movement of the fine adjustment changed the appearance to that hown in fig. 1, H, where there is only one obvious dark band. However, it eemed that the former pattern (fig. 1, G) was probably at the correct focus (i.e. on the greatest horizontal diameter), since it was the only one where there was a symmetrical change in the pattern on focusing both up and down.

Treatment of unfertilized eggs with trypsin prevents the elevation of the ertilization membrane when the eggs are fertilized (Runnström, Monné, and Broman, 1943). It has been suggested that trypsin might act by digesting way a vitelline membrane at the surface of unfertilized eggs which acts as a recursor of the fertilization membrane. If this happens, and if the pattern at the edge of the unfertilized egg is caused by a vitelline membrane, the edg of an egg treated with trypsin should look different from an untreated egg This, however, did not occur. Fig. 1, I shows the edge of an unfertilize *Paracentrotus* egg which had been immersed for 10 minutes in a 0·1% solutio of trypsin in sea water. It can be seen that the pattern at the edge is the same a that of the untreated egg (fig. 1, G).

The pattern at the edge of the fertilized eggs was very similar to that i unfertilized eggs. Fig. 1, J shows the edge of a *Paracentrotus* egg at the sperm aster stage in Ca-free sea-water (this removes the hyaline layer). The two mai rings are present at the same spacing (about 0·8μ) as before, and there are als subsidiary rings outside. The only difference is that the innermost ring is les clearly defined, as are all the subsidiary rings. The fertilization membrane ca be seen about 5μ outside the edge and, in normal sea-water, the hyaline laye would also have been visible. There was no change in the pattern at the edg during interphase and cleavage except for the growth of the hyaline layer o eggs in normal sea water.

Although they have not been illustrated, similar patterns occurred at th edge of *Arbacia* eggs. There was no change after trypsin treatment, and onl a slight change in the contrast of the rings after fertilization.

It was clear from these observations that there was no layer visible in th living uncentrifuged egg which was of sufficient thickness to be the cortex.

Sections

Sections of normal eggs were prepared and examined in the same way a with the centrifuged eggs (p. 112). The most obvious feature at the edge o unfertilized eggs fixed in Bouin was a thin pale layer. This occurred in both *Paracentrotus* and *Arbacia* (fig. 2, K and L). It was far less sensitive to change in focus and changes of the condenser-iris than the edge pattern in living eggs and it appeared to be a real structure rather than a diffraction pattern. Its thickness, corrected for shrinkage, was about 0·8μ. This is not, however, a very reliable figure since it represents a measured thickness of about 0·6μ and even with the best oil-immersion objectives this separation is getting too low for accurate measurement. This layer, though considerably paler than the cytoplasm, was not a clear structureless membrane. It appeared as though there were a number of spaces in it which could have been left by the disappearance of granules.

Unfertilized eggs which had been treated for 10 minutes with 0·1% trypsin showed exactly the same layer when fixed in Bouin as the normal eggs; so also did unfertilized eggs whose jelly had been removed by acid treatment.

Unfertilized eggs which had been fixed in Flemming did not show this pale layer (fig. 1, M). The only trace of structure at the edge of these eggs was that in some cases the whole cortex could just be made out as a thicker layer with a slightly different appearance from the rest of the cytoplasm. There was no sign of any layer of cortical granules which differed from the granules in the rest of the egg.

The pale layer could not be seen after Bouin fixation either in the normal fertilized eggs of both species (fig. 1, N, O, P), or in fertilized eggs where the elevation of the fertilization membrane had been prevented by pre-treatment with trypsin. Fertilized *Paracentrotus* eggs showed no boundary layer of any sort, but fertilized *Arbacia* eggs showed a relatively thick layer at the edge (fig. 1, O and P) with a vacuolar appearance. This layer is almost certainly the cortex, the vacuoles being the site of pigment granules which have disappeared during fixation and staining. Measurements on this layer with 25 eggs fixed in Flemming gave an average corrected thickness of $1{\cdot}13\mu$ (S.D. = 0·18) for the sperm aster stage, and 1·50 (S.D. = 0·31) for early cleavage. There was a definite thickening in the layer at cleavage, but there were no obvious signs of different thicknesses in different parts of a cleaving egg (fig. 1, P).

DISCUSSION

The cortex

It is impossible to see the cortex in a normal living sea-urchin egg under an ordinary microscope, and it is difficult to see it in stained sections except with certain species such as *Arbacia*. The most obvious difference between the cortex and the cytoplasm is that the cortex is stiffer and more gelated, and it is therefore not surprising that the first definite proof of its existence came from the microdissection experiments of Chambers (1917). An elegant method of demonstrating the cortex is to introduce an oil drop (Chambers, 1938*a*, 1938*b*) or a metallic particle (Seifriz, 1924) into the egg, and then show that when the foreign body is moved towards the edge it is held away from the outer side of the cell membrane by a solid layer of appreciable thickness. No figures have been given from these experiments for the thickness of this layer except by Chambers (1938*b*) in the case of a cleaving egg of *Lytechinus*. He found that an injected oil drop was indented 5μ ahead of the advancing furrow, which is a much greater value for the cortical thickness than the results in the present paper would indicate. The difference may be due to the different species of sea-urchin, but it is also possible that Chambers's observation may be an over-estimate since it is very difficult to determine accurately the position of the edge of the furrow in a living egg in late cleavage.

Microdissection experiments are the most direct way of showing the existence of the cortex, but they are not so suitable for measurements of its thickness. It is easier and probably more accurate to use the indirect method of measuring the thickness of the granular layer left at the egg surface after centrifugation. There can be little doubt that this layer is the cortex, and that the granules are held in place by the comparative rigidity of the cortical gel. This method of demonstrating the cortex has been used by a number of workers from Lillie (1906) onwards, but in no case has the thickness of the cortex been measured carefully. It is, however, apparent from the photographs of Motomura (1935) that the thickness of the cortex in stained sections of the unfertilized eggs of three Japanese echinoderms is about $1\text{–}1{\cdot}5\mu$.

The cortex can also be distinguished from the cytoplasm by two optical characters: it is birefringent, and it has different light-scattering properties. Monroy and Oddo (1946) have given values for the cortical thickness from measurements of the breadth of the birefringence pattern at the edge of a number of sea-urchin eggs. Although these values (1·2–1·8μ) agree with the results in this paper, this method is likely to give inaccurate answers unless the problem of diffraction and out-of-focus images has been considered (Mitchison, 1953*a*). Runnström and Monné (1944) have given a figure of 1μ for the cortical thickness from birefringence measurements. This result, however, is erroneous because the thickness they have calculated is the amount of the cortex traversed by the light ray tangential to the inner edge; this is a much larger figure than the normal radial thickness.

Turning to the results in this paper, it can be seen from table 1 that the cortical thickness in the sections of centrifuged eggs lies between 1·13 and 1·35μ. There is no significant difference either between the two species of sea-urchin or between the three stages of development. In the case of the living eggs, however, the cortex was appreciably thicker (2μ±0·5μ) in both sea-urchins and at all stages. In attempting to reconcile these values, it must be remembered that the measurements on the living eggs are considerably less accurate than those on the sections. The cortical layer curves inwards both above and below the focal plane and tends to obscure the inner edge, while the outer edge is difficult to separate from the diffraction patterns which are prominent in water. At the same time, there is the problem of shrinkage in the fixed sections. The figures for cortical thickness have been corrected for the total shrinkage shown by the eggs, but it is quite possible that the cortex shrinks by a greater amount. Taking these factors into consideration, and remembering that the microscope has a finite resolving power (0·25–0·35μ in this case), it seems reasonable to take a figure of 1·5μ for the approximate thickness of the cortex at all stages of development up to the first cleavage. It is obviously impossible to set any accurate limits on this figure, but it seems unlikely that the cortex is greater than 2μ or less than 1μ in thickness.

In most of the European sea-urchins the cortex is invisible in stained sections of normal eggs, but in the fertilized eggs of *Arbacia* there is a vacuolated cortical layer. The vacuoles are presumably caused by the disappearance on fixation of the large red pigment granules. It is, in passing, a little curious that a similar layer is not present in the unfertilized egg which also contains these granules in the surface, though in smaller numbers. At the sperm aster stage, the thickness of this layer (1·13μ) was less than that in the centrifuged eggs (1·35μ), while at cleavage it was greater (1·50μ compared with 1·17μ). It seems unlikely, however, that these differences correspond to any appreciable changes in the thickness of the living cortex in view of the absence of such changes in the centrifuged eggs.

It should be mentioned at this point that the relatively large size of the pigment granules is a possible source of error in the measurements on fertilized *Arbacia* eggs. What is being measured in this case is really a single layer of pigment granules, and it might be argued that they were only attached to the inner edge of a much thinner cortex. Although this is a possible objection, it seems more likely that the granules must be well embedded in the cortex if they are to resist the centrifugal force, and that the cortex therefore must be nearly as thick as the diameter of the granules.

There are three further points about these experiments which should be stressed. First, there were no obvious differences in the cortical thickness between different parts of the surface of cleaving eggs. This was apparent in the sections of normal *Arbacia* eggs (fig. 1, P). Also, if there had been such differences, there should have been a large variation in the cortical thickness of the centrifuged cleaving eggs, since the eggs stratified independently of the cleavage plane. It can be seen from table 1 that the standard deviations at cleavage are no greater than at other stages. Secondly, there was no sign in the sections of any differentiated region in the cytoplasm ahead of the cleavage furrow similar to that found in amphibian eggs by Selman and Waddington (1955). Thirdly, there was no difference in the cortical thickness in living eggs which had been centrifuged at different speeds. This argues against the suggestion of Chambers (1917) that the gelated cortex changes gradually into fluid cytoplasm at its inner edge.

The vitelline membrane

There has been discussion for many years about the nature and existence of a vitelline membrane in sea-urchin eggs. The vitelline membrane would be defined as a zone or membrane which can be distinguished optically at the outer surface of an unfertilized egg and which gives rise to the fertilization membrane when the egg is fertilized or activated. It has been shown on a number of occasions that a thin membrane, which has been called the vitelline membrane, can be separated from the surface of unfertilized eggs either by mechanical distortion of the eggs (e.g. by crushing (Herbst, 1893); by shrinking in hypertonic solutions (Runnström, Monné, and Broman, 1943), or by microdissection (Kite, 1912; Chambers, 1921)). These experiments, however, only show that a membrane *can* be produced by the surface of an unfertilized egg, and not that there is a vitelline membrane in a normal untreated egg. It must be remembered also that these mechanical treatments can activate eggs, so it is quite possible that what were called vitelline membranes were in reality fertilization membranes.

The critical question is whether or not a vitelline membrane can be seen at the surface of a normal unfertilized egg. The earlier evidence is conflicting; some workers say that they can see a thin surface membrane (Selenka, 1878; Herbst, 1893), others that it is very difficult or impossible to see (Heilbrunn, 1913; Glaser, 1913; Chambers, 1921). It may of course be easier to see in some species of sea-urchin than in others, but one is inclined to suspect observations like those of Kite (1912), who finds a vitelline membrane $1{\cdot}9\mu$ in thickness which is visible only when the condenser is stopped down. This is just the condition which produces prominent diffraction rings at the egg surface.

The observations in this paper have been made under the best optical conditions and they reveal all that is likely to be seen with an ordinary microscope. It is, however, difficult to give any adequate interpretation of the pattern at the edge of living eggs in water. At first sight it would seem that the two innermost rings visible at the edge of the unfertilized eggs defined a vitelline membrane, but this is unlikely in view of the fact that the rings also appeared in the fertilized eggs. One point that must be borne in mind is that pure diffraction phenomena at the edge may give the effect of a membrane. If the edge of a light-absorbing object is examined with a stopped-down condenser, the two central diffraction bands will be twice as far apart as the other bands and will give the appearance of a separate membrane at the edge. This can be shown from the fact that the intensity at a light-absorbing edge with coherent illumination varies as $[\pi/2 \pm Si(ax)]^2$, where $a = 2\pi(\text{N.A.})/\lambda$, Si is the sine integral, x is the distance from the edge, N.A. is the numerical aperture of the objective, and λ is the wavelength of the light. This is not of course an adequate model for the edge of the egg (which is mainly a phase object, curving away above and below the focus, and examined with a wide condenser iris), but it does suggest that thin membranes at the edge of objects may be optical artifacts. There is at the moment no optical theory that deals with the diffraction phenomena at the edge of such a complex object as a sea-urchin egg in water, and the only guide that can be given to the microscopist is to regard with suspicion any 'membrane' which is bordered by diffraction rings of the same order of size and of contrast. Such diffraction rings could be seen at the edge of the living eggs; so both for this reason and because the patterns did not correspond with the appearance of the sections, it would be unjustified to draw from these patterns any conclusions about the presence of a surface membrane.

The only other visible structure which might be the vitelline membrane is the pale layer in the sections of unfertilized eggs fixed in Bouin. There is little doubt that this layer represents a real structure and, since it is absent in fertilized eggs, it would not be unreasonable at first sight to assume that it is the vitelline membrane. There are, however, three points about it which argue against this conclusion. In the first place, there is the fact that its thickness is of the order of $0{\cdot}8\,\mu$ or 8,000 Å. I have shown in another paper (Mitchison, 1953*b*) that the thickness of the fertilization membrane is about 500 Å. Allowing for the area of the fertilization membrane being up to double that of the egg surface, the maximum thickness of the vitelline membrane should be only 1,000 Å. Even this is likely to be an overestimate, since it is thought that some of the material of the fertilization membrane comes from granules which leave the egg surface after fertilization. This suggests that the 8,000 Å pale layer is too thick to be the vitelline membrane, which should only be about one-tenth of this figure. The second reason for suggesting that the pale layer is not the vitelline membrane is the effect of trypsin treatment. The two most likely modes of action of trypsin are either that it digests away the vitelline membrane or that it so disorganizes the vitelline membrane that it cannot form a fertilization membrane. In the first case the vitelline membrane should disappear from the surface of the unfertilized egg after trypsin treatment, and in the second the vitelline membrane should be visible on the surface of a trypsin-treated egg both before and *after* fertilization. The behaviour of the pale ayer, however, does not fit either of these alternatives. It is visible in the unfertilized egg after trypsin treatment, but disappears when a trypsin-treated egg is fertilized. This third argument against the pale layer being the vitelline membrane is that it is absent in eggs fixed in Flemming. There is no reason to suppose that it would be preserved by a bad cytological fixative and destroyed by a good one.

An alternative explanation of the pale layer is that it is the whole or part of the jelly which has been condensed down on the egg surface during fixation. This seems unlikely for two reasons. First, the layer is still present after the eggs have been treated with acid, which is usually believed to remove all the elly. Secondly, the layer appears to have an internal structure and a sharply defined outer edge. A condensed layer from the jelly, on the other hand, would probably have little internal structure and no sharp edge.

The last and most likely explanation is that the pale layer is the outer layer of the cortex which normally contains the cortical granules. These granules could not be distinguished from the other cytoplasmic granules in the sections which were examined (though the technique used—Flemming fixation and iron haematoxylin staining—was that recommended by Runnström, Monné, and Wicklund, 1946), but they undoubtedly exist, and have recently been photographed very elegantly in living eggs by Endo (1952). They are relatively unstable and break down at fertilization, so it is not surprising that they are destroyed by Bouin and leave a vacuolated layer, but are preserved by Flemming which is a better cytological fixative.

This discussion can be summarized in the form of a diagram of the suggested structure of the cortex of an unfertilized egg (fig. 2). The pale layer occurs at the outer edge of the egg and this indicates that the cortical granules lie in the outer layer of the cortex (about $0{\cdot}8\,\mu$ thick) directly below the plasma membrane which surrounds the egg (fig. 2, A). Below them lies the rest of the gelated granular cortex to make a total cortical thickness of about $1{\cdot}5\,\mu$. In a normal egg the cortex is indistinguishable from the fluid granular cytoplasm, but in a centrifuged egg the cytoplasm is cleared of granules at the centripetal end and the cortex becomes visible (fig. 2, B). In an egg fixed in Bouin, the cortical granules are destroyed, and the outer layer which they occupied becomes visible as the pale layer (fig. 2, C).

This suggested structure for the cell membrane or cortex is very similar to that proposed by Runnström (1948) and Endo (1952) except that they place a vitelline membrane outside the plasma membrane. The work in this paper indicates that there is no vitelline membrane which can be distinguished by the light microscope, but it does not rule out the possibility that there is a membrane below the limit of resolution. Indeed, if there were a vitelline membrane, one would expect it to be below the resolving limit since it has been argued above that its thickness should not be greater than 1,000 Å. This clearly a matter for the electron microscope, but so far the evidence fro thin sections prepared for the electron microscope is against the existence a vitelline membrane. Neither McCulloch (1952*a*) nor Hillier, Lansing, an Rosenthal (1952) found any obvious vitelline membrane. The latter worke found in *Arbacia* eggs a thin surface membrane with papillae on it, bu

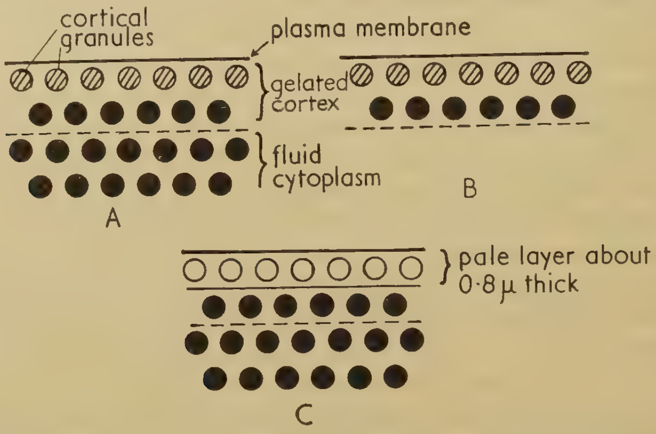

FIG. 2. Diagrams of cortical structure in unfertilized eggs. A, living egg or egg fixed in Flem ming. B, centripetal end of centrifuged egg, living or fixed in Flemming. C, egg fixed in Bouin

these papillae were still present in fertilized eggs. Both McCulloch (1952*b* and Cheney and Lansing (1955) show photographs of the cortical granule lying directly below a very thin surface membrane.

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The Peri-renal Tissue of *Protopterus*
A Contribution to the History of the Adrenal

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With three plates (figs. 3, 5 and 8)

SUMMARY

The animal studied was the lungfish *Protopterus aethiopicus*; the investigation was histological and cytochemical, the material being freshly taken from East African lakes.

The peri-renal tissue is a substantial organ which, because it surrounds the kidney, extends through the greater part of the body cavity. Those who have investigated its structure in recent years have considered it to be a haematopoietic organ.

A wide variety of histological, histochemical, and haematological techniques was used. The organ is shown to contain five characteristic kinds of tissue; these are named: lipid, endothelial, pigmented, round-cell, and steroid tissue respectively.

The lipid tissue is characterized by cytoplasmic liposomes—droplets containing phospholipid. In one type of cell, the 'small lipid cell', which may be related to the eosinophil granulocytes of the blood, the droplets have a relatively small diameter. In a second type, the 'large lipid cell', the droplets grow to a much larger diameter, and show evidence of cycles of activity involving increase and disappearance of the phospholipid. Histochemical interpretations are made difficult by the finding that this phospholipid does not colour with Sudan black.

The endothelial tissue is composed of phagocytic (macrophage) cells some of which are elongated and line vascular sinusoids while others are spherical and contain larger inclusions. These inclusions have some lipid characteristics and may be a manifestation of the translocation of lipid within the organ.

The pigmented tissue is composed of melanophores and of a second, round, type of cell which shows evidence of relationship with the macrophage system. Both kinds of cell contain 'melanin'; the second also has inclusions with different histochemical properties.

The round-cell tissue, found in nodules, has a general resemblance to lymphoid tissue. It is doubtful whether its cells are lymphocytes, but it has not been found possible to characterize them in any other way.

The steroid tissue is composed of cells which react positively to the histochemical tests comprised in the 'steroid repertoire', as used in the study of the mammalian adrenal.

Attention is drawn to technical difficulties which may have confused the study of the peri-renal tissue as a haematopoietic organ. The connexion between the lipid tissue and circulating granulocytes has features which distinguish it from the processes of granulocyte formation in the spleen.

There are strong indications of an evolutionary connexion between peri-renal tissue and the cortex of the adrenal gland of higher animals. The lungfish may offer the best starting-point for the study of the comparative histology of the adrenal of tetrapods. The association between lipid, steroid, haematopoietic, macrophage, and pigment tissue has parallels in function in the complex interrelations of the mammalian adrenal.

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INTRODUCTION

THE structure which appears to be the kidney in a dissected lungfish i found, when sectioned, to consist only in part of renal tissue. The tru kidney forms a central mass, and is surrounded by a tissue of different histological characteristics. This peri-renal tissue has been mentioned by many o those who have studied the micro-anatomy of the lungfish. Three of the mos recent accounts of its structure have been by workers who believed that i forms a haematopoietic organ (Jordan and Speidel, 1931; Dustin, 1934 Champy and Louvel, 1939). Dustin suggested also that it may, in part, have a endocrine function. Holmes (1950) showed that some of its component cell react positively to a histochemical test indicative of the presence of steroi substances, and that a substantial part of the tissue consists of cells loade with droplets containing phospholipid. He suggested that these similarities t the cells of the mammalian adrenal cortex justify the hypothesis that the peri-renal tissue and the adrenal cortex have a common phylogenetic origin.

Since the lungfish kidneys extend through the greater part of the body-cavity the total mass of the peri-renal tissue is considerable. The question o the structure and function of this complex tissue, large enough to qualify a an 'organ', cannot fail to be of interest, and the view that it is 'simply' an haematopoietic organ does not cover all the facts of its structure. Furthermore there are such profound divergences of opinion between the three recen studies of lungfish haematopoiesis that a re-examination of the question cannot be avoided.

This present account is of the normal peri-renal tissue; an account of the results of an experimental investigation will be separately published (Moorhouse, 1956). No reference will be made to the adjacent tissue which covers the gonad; this appears to have some characters in common with the peri-renal tissue, but to be less diverse in histological structure.

COLLECTION OF MATERIAL

The abundant supply of normal material necessary for cytological research, and the laboratory facilities for the care of experimental animals, necessitated a stay in Africa. This part of the work was done in the laboratory of the East

African Fisheries Research Organization at Jinja, Uganda, on the northern hore of Lake Victoria.

The margin of the lake, within half a mile of Jinja, provided a steady supply of lungfish. The fish were living in the wet mud between the dry land and the mats of papyrus which extend out for some yards into the waters along the margin. They were caught by digging out lumps of the loose mud and flinging hem on the bank; the fish were extremely active and had to be quickly capured; it should be emphasized that these were not 'encysted' animals, but ndividuals which, in day-time at least, wriggle about in the mud, which is rich in the worms and molluscs on which they feed. The animals obtained in his way were all 'medium-sized' individuals, ranging from 130 to 300 mm in otal length.

Smaller individuals, with well-developed external gills, were sometimes obtained in the same way. They were also caught by dragging a small fishing-net through the shallow water amongst the papyrus. The smallest we obtained had a total length of 49 mm and its external gills were still conspicuously feathered. Animals about 50–55 mm had lost the feathering, and some specimens of 65 mm showed no external gills to naked-eye examination.

We were particularly anxious to obtain even younger stages, for embryological studies, but failed to do so. A good deal of information is available about the breeding nests of West African lungfish, from investigations in the Gambia river. It is in these nests that the earliest development takes place. But in spite of prolonged search we could not find similar nests on the shores of Lake Victoria, nor in the masses of floating papyrus which we explored by boat. Johnels and Svensson (1954) have given a thorough survey of existing information on the natural history of the lungfish, and have added much that is new from observations in the Gambia. But they remark that no reliable account of the breeding nests of the East African forms is available, and state that only one man has seen the young of *Protopterus aethiopicus*. It can be concluded from their account that the fish develops to a length of 49 mm (our smallest form) in a matter of days. Neither the European biologists nor the Africans at Jinja could help us to find the youngest stages.

We saw lungfish of a length of more than 1 m, but animals of this size were not found inshore. In Lake Victoria they evidently live in deeper water and can be seen there from boats, coming to take air at the surface. We attempted to catch these larger specimens by trawling, but very few were obtained and these were badly damaged. Individuals caught in gill-nets were also damaged, and had often been dead for some time, presumably from lack of air. One of us (D. E. M.) was able to visit Lake George, as a guest of the Uganda Fish Marketing Corporation. This organization lands hundreds of large lungfish every day, caught by long-line fishing, and provided us with our largest specimens. There seems no doubt that fish of this size are far more common in Lake George (and in Lake Edward) than in Victoria, a fact which may be correlated with the far denser population of phytoplankton found in the former lakes.

It is necessary to say a word on the specific identity of our specimens of

Protopterus. All specimens of lungfish recorded from lakes Victoria and E ward have been assigned to the species *aethiopicus*, and we have assumed th we are dealing with this single species. At the same time, the most recent stud of the systematics of East African lungfish has shown how imperfect ou knowledge is (Trewavas, 1954). Thus Dr. Trewavas has found it necessary t reassert the specific distinctness of *P. amphibius*, as a third East African for and thinks it possible that some subspecific characters may separate the Ea African *P. annectens* from the form of this species found in its typical localit the Gambia. But we can find no grounds for doubting that our material ha been throughout of the fish at present known as *P. aethiopicus*. (We are fortu nate in possessing a specimen of *P. amphibius* presented to D. E. M. by Lor Richard Percy, from his collection made in Kenya.)

METHODS

The cytoplasmic inclusions, in the form of droplets, which are characteristi of so many of the peri-renal cells, are demonstrable by staining in paraffi sections, even after the use of fixatives containing protein precipitants. Henc we were able to use serial sections to establish the general characteristics an arrangements of the tissue. For this purpose we tested many fixatives an came to prefer formaldehyde / mercuric chloride (10 ml 40% formaldehyd + 90 ml saturated aqueous mercuric chloride), and Helly's mixture. Th former fixative gave excellent results when the sections were stained wit haemalum and the carbol / chromotrope of Lendrum (1947); or with th 'Azan' technique. Helly's mixture was preferable when the sections wer stained with azure II / eosin or by Pasini's technique (both methods as de scribed by Pickles, 1950). Each of these procedures has its particular advan tages, and all were employed.

The procedure employed for the demonstration of mitochondria consiste in fixation in Helly's fluid followed by dichromate mordanting of the block This gave excellent fixation, and preservation of almost all cytoplasmic in clusions. Such sections, stained by Metzner's method for mitochondria, wer of the greatest value.

The apparent similarity between granular leucocytes and many peri-rena cells necessitated, for further analysis, the use of some special haematologica staining methods. The technique of Pasini and the carbol / chromotrope an azure II / eosin methods have themselves been found of use by students o haematopoietic organs; in order to compare the peri-renal cells with those o the circulating blood and with the contents of the spleen we have also applie Romanowsky staining mixtures to these tissues in the form of smears and impression preparations. After testing many procedures we preferred, and chiefly used, Leishman's stain (made up from powder, B.D.H.) applied to air-drie smears and to impressions either air-dried or fixed in the vapour of formaldehyde

The larger part of our work has, however, been devoted to the application o histochemical methods to the tissue under consideration; we considere that, of the techniques available to us, it was these which were most likely t lead to a description of structure which would be a useful basis for the studies of function which are still to come.

When histochemical methods are used to determine the structure and chemical composition of a single kind of cell, at a single stage of development, in an animal whose life history is known, the problem can be rigorously defined and the methods applied so as to extract information to the limit of their potentialities. The problem is more complicated, the investigation more elaborate, and the conclusions less precise in a case such as ours.

The largest lungfish we investigated had a length of about 0·75 m, and the peri-renal tissue extends over half the length of the fish. No account of it yet given includes evidence of its organization in the third dimension; that is to say, that we do not know whether statements about its structure apply equally at every level in transverse section or only to an arbitrarily chosen length of a few millimetres. Furthermore, the dimension of time must be of the greatest importance in a tissue whose adrenal-gonad relationships undoubtedly link it with seasonal cycles and even more complex rhythms.

Very little is known of the rate of growth and of the natural history of breeding in *Protopterus*. The scientists of E.A.F.R.O. could tell us nothing new; the latest contribution, from West Africa, deals largely with the youngest stages and with the habit of 'aestivation' in the dry season (Johnels and Svensson, 1954). Since the level of Lake Victoria does not normally show substantial seasonal fluctuations it is likely that the greater part of the lungfish population of the lake does not undergo regular 'aestivation'. For, as Johnels and Svensson show, this activity is a response to the drying up of large areas of water, which leaves the fishes stranded.

We attempted to meet these limitations by the following kind of procedure. The main body of our observations was made on fishes of a total length of from 130 to 250 mm taken from the lake-shore habitat already described. The sex of each individual was determined, but we found no difference in the peri-renal tissue which could be correlated with this variable. In the apparent degree of maturity of the gonad our samples showed great variation within the group at all times of the year (our collecting point was approximately 0° 30′ N. of the equator). We also studied very small and very large individuals, though in much smaller numbers. The former group consisted of specimens of a total length of from 40 to 65 mm still having conspicuous external gills. In these animals the peri-renal tissue has, in much of its extent, a thickness of one or two cells only, but the major cell types characteristic of the adult have already appeared.

Preliminary studies indicated that this side of the problem would lie chiefly in the determination of the nature and distribution of the lipid inclusions of the peri-renal cells. Complete histochemical surveys, at levels not more than 4 mm apart in any one animal, were carried out by the following methods:

1. Sudan black for lipids, according to the procedure of Baker (1944).
2. Acid haematein for phospholipids, with controls, according to Baker (1946).

3. 2:4-dinitrophenylhydrazine for steroids (Yoffey and Baxter, 1949).
4. Tetrazotized diorthoanisidine for steroids (Ashbel and Seligman, 1949).

Results given by these methods were from time to time compared with parallel procedures, as, for example, the use of acetylated Sudan black (Casselman, 1954), Sudan IV, Schultz's modification of Liebermann's test, and the digitonine reaction. Nile blue sulphate was not used extensively, since in our first trials we found that it did not establish any differentiation amongst the peri-renal cells. The plasmal reaction proved unsatisfactory for our purposes, whether applied to fresh or freshly fixed tissue (Cain, 1949; Nicander, 1951): the cytoplasmic inclusions were so seriously distorted or destroyed as to be unfit for study—though it must be admitted that, since we needed fresh material, this technique had to be used in Africa, where conditions were not ideal.

These basic techniques were supplemented by many others, which we shall not list here; it seems better to refer to them in the context in which results are being described. In all cases the reference given is to the author whose recommended procedure was followed exactly, so that we do not necessarily refer to the inventor of any particular test.

Throughout this work we have taken the greatest care to select the material and treat it throughout with complete regard for the requirements of cytological research. The animals were killed by decapitation.

RESULTS

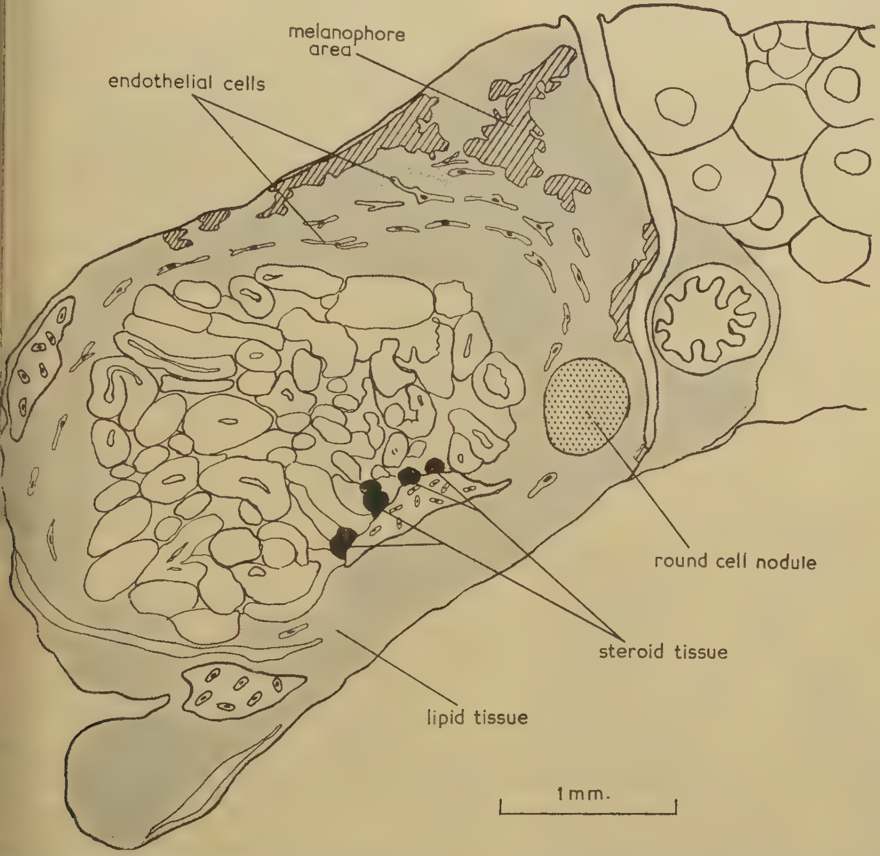
The organization of the peri-renal tissue

In this section we shall sketch out the general organization of the tissue and define the five special kinds of cell which characterize it. Further details of cytology and cytochemistry are given on pp. 144–50, in which the attempt is made to relate structure to function and to provide a basis for the further analysis of the tissue as an organ.

In the very young animals it can be seen that the peri-renal tissue is appearing as a layer, usually one cell thick, in the coelomic epithelium which suspends the kidneys and connects them, in the mid-line, to the sub-notochordal tissue, including the dorsal aorta. The gonad rudiment is present on each side at the outer edge of the kidney and is also invested by this epithelium. It is not possible to determine whether the specific cells of the tissue are in fact derivatives of the epithelium or whether they come from the underlying mesenchyme.

At later stages that part of the impending mesentery which encloses the kidney loses its anatomical distinctness, so that the peri-renal tissue is intimately applied to the kidney tissue on its inner side, not separated by any anatomical plane, and no layer of coelomic epithelium, or connective tissue sheet, can be stripped off from its outer surface.

The efferent renal vessels—that is, the posterior cardinal on the left and the vena cava on the right—lie with their main trunks near the surface of each idney, on the ventro-medial border, and are thus partly surrounded by peri-enal tissue. The whole tissue has a very rich vascular bed, and the walls of the apillaries are usually so thin that they are difficult to identify in sections, xcept when they can be located by a conveniently placed red corpuscle. Vhen leucocytes are seen it is always difficult, and often impossible, to deter-ine whether they are intravascular or free.



IG. 1. Diagram of a transverse section through one kidney of *Protopterus* (animal of total ngth 160 mm); a portion of the attached gonad is included on the right. The location of the rincipal zones of the peri-renal tissue is shown conventionally. Lipid tissue is shown by fine stippling.

The lipid tissue. This element of the peri-renal mass may be given a first, ough, characterization as the tissue which stands out most conspicuously in utine histological preparations. It does so because many of its component lls contain cytoplasmic droplets which stain intensely with dyes such as sin, acid fuchsin, azocarmine, and chromotrope (fig. 3, c). These are the lls which have been called 'eosinophils' by several workers. These eosino-hil inclusions, in many of the cells, give an intensely positive reaction to

Baker's acid haematein test for phospholipids (figs. 2; 3, A and B), and a negative in the pyridine-extracted controls. Since the word 'eosinophil' ha special connotation we think it better to speak of these as lipid cells, being cel which at some stage of their life history contain lipid droplets, or liposome which are distinguishable from mitochondria by their size and distribution

The characteristic cell has a nucleus of rounded shape, of diameter usual within 8–13μ. The extent of its cytoplasm varies very widely within a con tinuous series of forms; at its least extent it is a narrow coat round the nucleu

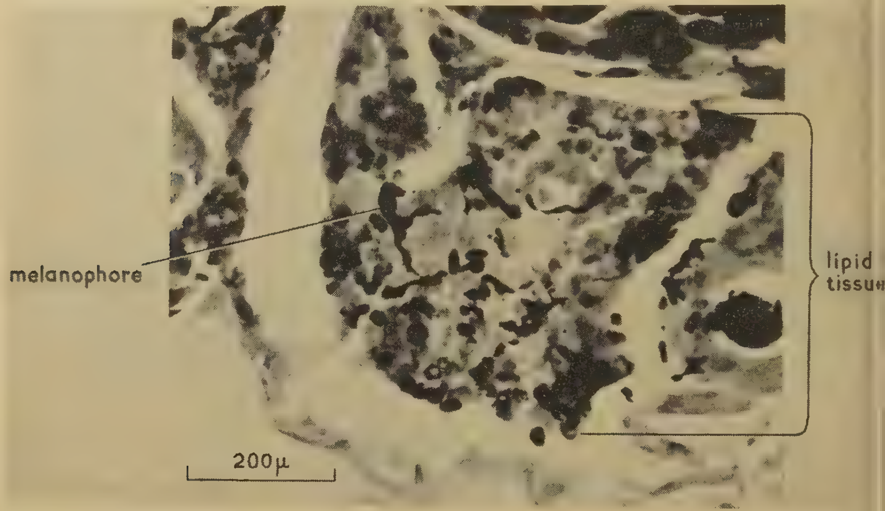


FIG. 2. Transverse section through a patch of lipid tissue. Acid haematein. The elongated branching cells are melanophores, which are not coloured by the dye. The other black-staine objects are large lipid cells, the stain being located in the cytoplasmic inclusions.

(fig. 3, D); when most extensive it forms a cell whose total diameter is mor than 30μ (fig. 3, A and B). Those cells which have the least cytoplasm contai no cytoplasmic inclusions; the rest contain spherical or ellipsoidal inclusion whose size and number increases with the extent of the cytoplasm and in manner indicating that there is a synthesis of phospholipid-containing materia during the life of the cell (fig. 3, A and B). At all stages of multiplication an growth these inclusions stain with dyes such as eosin and the carbol-chromo trope mixture. Acid haematein and mitochondrial preparations show, how ever, that this population of eosinophil inclusions is not a homogeneous on

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FIG. 3 (plate). Views of the lipid tissue.

A and B are photographs at two focal planes of an identical area in a section. Acid haematei The cell *X* is seen to contain droplets of various sizes, but none are as large as those in th fully formed large lipid cells.

C, lipid tissue, haemalum and carbol / chromotrope.

D, lipid tissue, Metzner's method for mitochondria; lipid cells at various stages of develop ment. The inclusions of the large lipid cells are so heavily stained that the cells appear as soli black masses in the photograph.

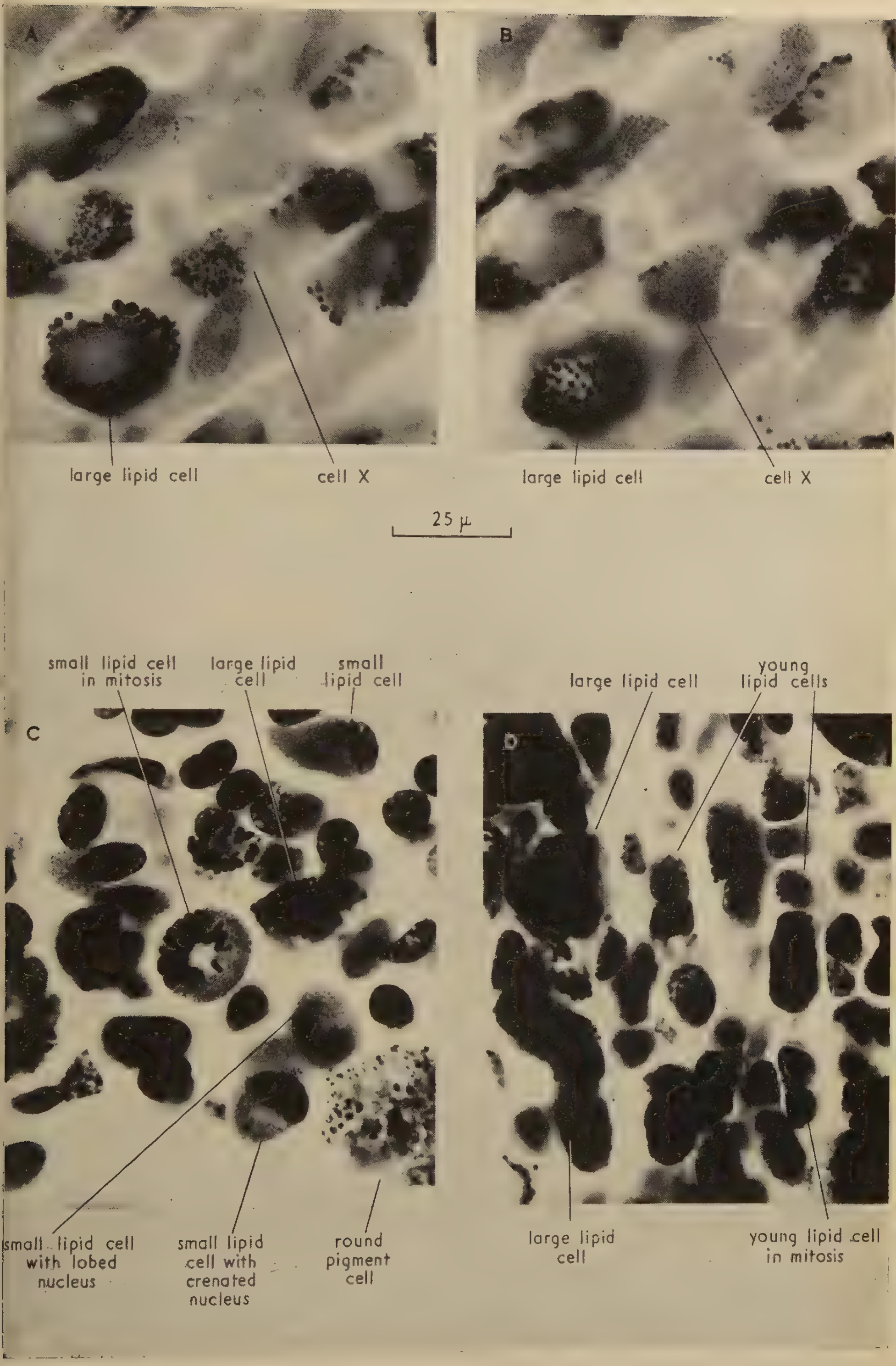

FIG. 3

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For in any given section of the lipid tissue a proportion of the largest cells contains inclusions which, though eosinophil, are negative in reaction when treated with the acid haematein and mitochondrial procedures. It is well known that, in many other tissues, these procedures stain bodies of similar chemical composition. It can be concluded that the liposomes of the lipid tissue are, at one stage in the life of the cell, so altered that by change or loss of phospholipid they cease to give these characteristic reactions. In such preparations the unstained liposomes appear as 'empty vacuoles'; at the same time their positive reaction to other stains suggests that the use of this phrase would be deceptive.

It would have been helpful had it been possible to analyse these changes in the liposomes by other methods from the repertoire of lipid histochemistry. The results with Sudan black, which we used as a routine throughout the investigation, proved paradoxical, though the paradox may ultimately be fruitful. In none of our preparations did the inclusions of the majority of lipid cells stain at all with Sudan black (or any other Sudan colorant), even in its acetylated form (Casselman, 1954), though Baker's test shows the presence of phospholipid. The possibility that the phospholipid is of so high a melting-point as to interfere with Sudan staining (Cain, 1950) was explored by staining sections at 37° C and 60° C in Sudan black solutions saturated at each of those temperatures. The results, however, were still negative.

The histochemistry of lipid complexes is as yet an undeveloped subject, and it is possible that the phospholipid in these cells is in the form of a complex which deprives it of its sudanophilia. We used both of the procedures of Berenbaum (1954), in which Sudan black is used under special conditions which are considered by their author to result in the staining of the lipid components of intracellular inclusions which are normally Sudan-negative. These procedures too did not stain the inclusions in the lipid cells. Unfortunately this problem had not arisen when we had access to sufficient fresh material to attempt to apply histochemical techniques after the use of biochemical methods for the unmasking of lipid from complexes.

The total mass of the lipid tissue relative to that of the kidney varies considerably between individual lungfish, and we have not been able to find any correlation between these variations and the size and sex of the animals; its mass, of course, increases in the first few months of life. Lipid cells of all sizes, with inclusions of corresponding size and number, and those with 'empty vacuoles' are intermingled in such a way as to suggest that they are members of one cell population, at different stages of their life. The major variation in the total mass of the tissue comes from variations in the number of the largest cell form—those with the most extensive cytoplasm, packed with inclusions. These full-sized cells are sometimes rounded in form, but, more usually, they become oblong rectangles; in this form, and lying end to end, they form an 'epithelium' of varying thickness on the outer surface of the peri-renal tissue (fig. 4). This layer is never sharply demarcated from the more irregularly arranged lipid cells below it. There are always rounded cells, also with inclusions, amongst the rectangular type, and the differences between these cells will be further discussed on p. 145. Lipid cells are also found in patches throughout the mesentery which invests the lungs and other adjacent viscera, and are particularly conspicuous close to the patches of chromaffin cells, near the dorsal aorta, which are considered to be the homologue of the adrenal medulla of higher forms (Holmes, 1950).

The cells of the peri-renal lipid tissue lie in a connective stroma best shown by Linder's reticulin technique and the tannin-ferric protein test of Salazar (Gomori, 1952) (fig. 4). Stains such as the aniline blue of Heidenhain's Azan show only scattered collagen fibres, but the first two methods reveal that the connective system is continuous throughout the tissue, separating every cell from its neighbour, though often by the finest of layers. Four or five rounded cells may often be seen grouped in a lobule, with a coarser reticulum investing the group. We hoped to give a more precise description of this tissue in three-dimensional terms, but the very thick sections necessary to contain more than four or five of these large cells in their depth proved too dense for microscopical study, even when unstained.

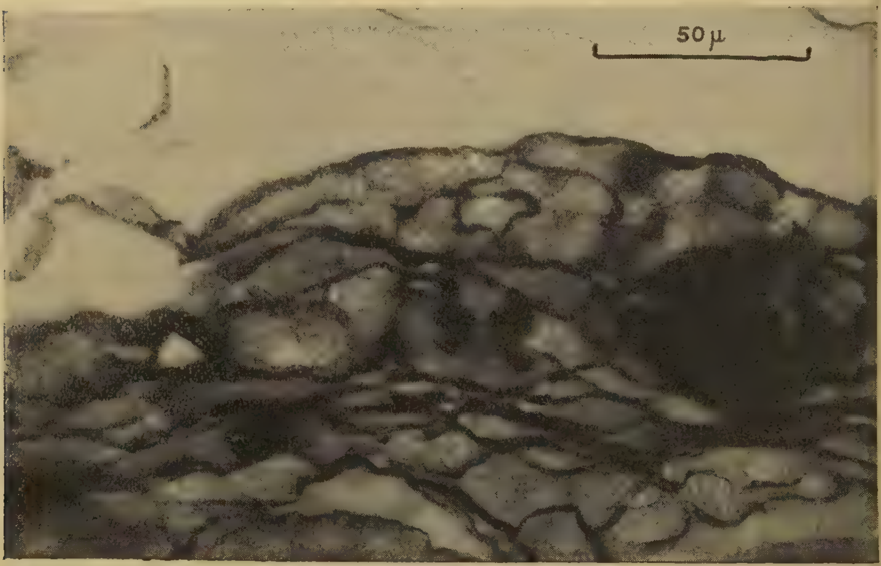

FIG. 4. Lipid tissue, Salazar's protein test. The connective stroma between the lipid cells is shown. The nuclei of small lipid cells which have a crenated nucleus have stained intensely, others are uncoloured.

The endothelial phagocytes. Each of a group of normal animals was injected with a freshly prepared solution of trypan blue in sterile normal saline of a concentration of 1%. The injection was made through the ventral body-wall, in the mid-line, at a point 4 cm posterior to the heart; it was carried out so that part of the dye solution was forced out into the muscles, the rest emptied into the body-cavity. The animals showed no ill effects, and were killed 24 hours later, and the viscera fixed in formaldehyde / mercuric chloride. Paraffin sections were examined unstained, and with various counterstains.

This procedure picks out clearly the second histological element of the peri-renal tissue, composed of phagocytic cells which take up particles of the dye in substantial amounts.

Many of the cells in this macrophage system can be seen to form the walls of a rich capillary network, which can thus be regarded as a sinusoidal system (fig. 5, A). It is most conspicuous as a band between the outer mass of lipid tissue and the kidney (fig. 1), but its outer edge merges into the lipid tissue and, in fact, the phagocytic cells are widespread throughout the peri-renal mass. They appear to be absent, however, from the zone in which the lipid cells, in cubical form, are arranged in epithelioid cordons and from the 'lymphoid' nodules and steroid areas which are shortly to be described. They are already present in a 60 mm animal, and occur at all levels of the peri-renal tissue of the adult. The phagocytic cells can readily be identified in sections stained by Pasini's method, and after haematoxylin in certain forms, and mitochondrial staining, for their cytoplasm normally contains many small rounded inclusions (fig. 5, A). These are found throughout the cytoplasm, but not necessarily uniformly distributed. They are not distinctly coloured by the acid haematein technique. They may perhaps be regarded as mitochondria, but they are rather large, and stain more readily with haematoxylin than these bodies usually do.

Not all the macrophage cells are thin and elongated as evident endothelial cells; various forms are found between this type and some with a round nucleus and rounded cell outline (fig. 5, B). These rounded cells are identical with the others in the presence of 'mitochondria', and in their capacity to ingest trypan blue particles. However, as is shown in fig. 5, B, they contain other larger inclusions, whose nature is discussed on p. 148.

Acid haematein preparations show the presence, amongst the sinusoidal cells, of a number of round cells with uniform cytoplasmic droplets containing phospholipid. Some, at least, of these are granular leucocytes circulating within the sinusoids; others belong to the category of lipid cells.

Pigment cells. The presence of pigmented cells in the peri-renal tissue is responsible for the brown and black speckling of the dissected 'kidney' and has been noticed by all who have studied its histology, for the colour remains visible after all histological procedures. One kind of these cells undoubtedly deserves the name of melanophores (Dustin, 1934). They lie amongst the lipid tissue, and have a dendritic form (figs. 2 and 5, C); their shape is best seen in longitudinal sections, since most of their ramifying processes extend along the antero-posterior axis of the animal's body. Cells of identical appearance are widely distributed in the viscera of the lungfish, being particularly numerous within the pancreas. The intracellular pigment is in the form of dark brown or black granules, of uniform size and uniformly distributed (fig. 5, C). The Masson–Fontana test (Pearse, 1954) often shows the granules to be argentaffin, though in many preparations these are naturally so black in colour that there is no significant darkening on silver treatment. Treatment with 1% aqueous bromine for 24 hours bleaches them to a pale yellow, and strong nitric acid treatment for the same period leaves them pale brown. The inclusions give no lipid staining reactions, and no part of the cell gives a positive reaction to the periodic acid / Schiff (PAS) test. They show no fluorescence in u.-v. light and have no other characteristics suggesting that the pigment is of any other kind than the group 'melanins'. However, 20 volume H_2O_2 applied for 24 hours to paraffin sections fails to bleach them completely, and we have not been able to investigate them with DOPA procedures, since the question of their nature had not arisen when we had access to fresh material in Africa.

There is a second and quite distinct type of pigment cell, which has a much more rounded form and never extends into dendritic processes (figs. 3, C, 5, D). Most of these cells lie farther in from the surface of the peri-renal tissue, being scattered between the kidney and the outer layers of lipid cells, and thus intimately mixed with both lipid and endothelial tissues. These round cells have two kinds of inclusion. First there are yellowish-brown, dark brown, or black particles which are different from those in the dendritic cells in being of assorted sizes. In addition there are numerous droplets in the cytoplasm, pale yellow in unstained material, which are also of various sizes, but often much larger than the brown particles. The brown particles give the same histochemical reactions or absence of reaction as the inclusions in the melanophores and, in so far as this amounts to a histochemical specification, they must be regarded as melanic. The pale-coloured droplets, on the other hand, react strongly to the PAS procedure, and they are thus of a different nature. Since they are normally slightly coloured it becomes important to determine whether they belong to any of the recognized categories of pigmented material other than melanin, and the following analysis was carried out.

The argentaffin reaction is negative and there is no autofluorescence in u.-v., so that the inclusions do not fall into the category of 'argentaffin granules'. In order to determine whether they are haem pigments, Perls' acid ferrocyanide reaction was applied, and sections were subjected to microincineration (we are grateful to Mr. Bradbury for carrying out this last investigation). These tests show that droplets containing iron are occasionally found in the round pigment cells, but very occasionally, and it is clear that, although iron may sometimes be present in the cytoplasm it is not a constituent of the coloured bodies which are invariably found in considerable numbers.

The possibility was then considered that the bodies are coloured by hydrocarbons of the carotenoid series, dissolved in cytoplasmic lipid droplets. The positive PAS reaction leaves it possible that the inclusions are lipoidal; they

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FIG. 5 (plate). A and B, sinusoidal zone with endothelial phagocytes. Metzner's method for mitochondria. In A thin elongated endothelial cells contain uniform-sized inclusions of mitochondrial type. In B there are also rounded macrophages with various-sized inclusions. Most of the dark bodies are erythrocytes in sinusoids.

C, melanophores amongst lipid tissue, acid haematein.

D, round pigment cell amongst lipid tissue, acid haematein.

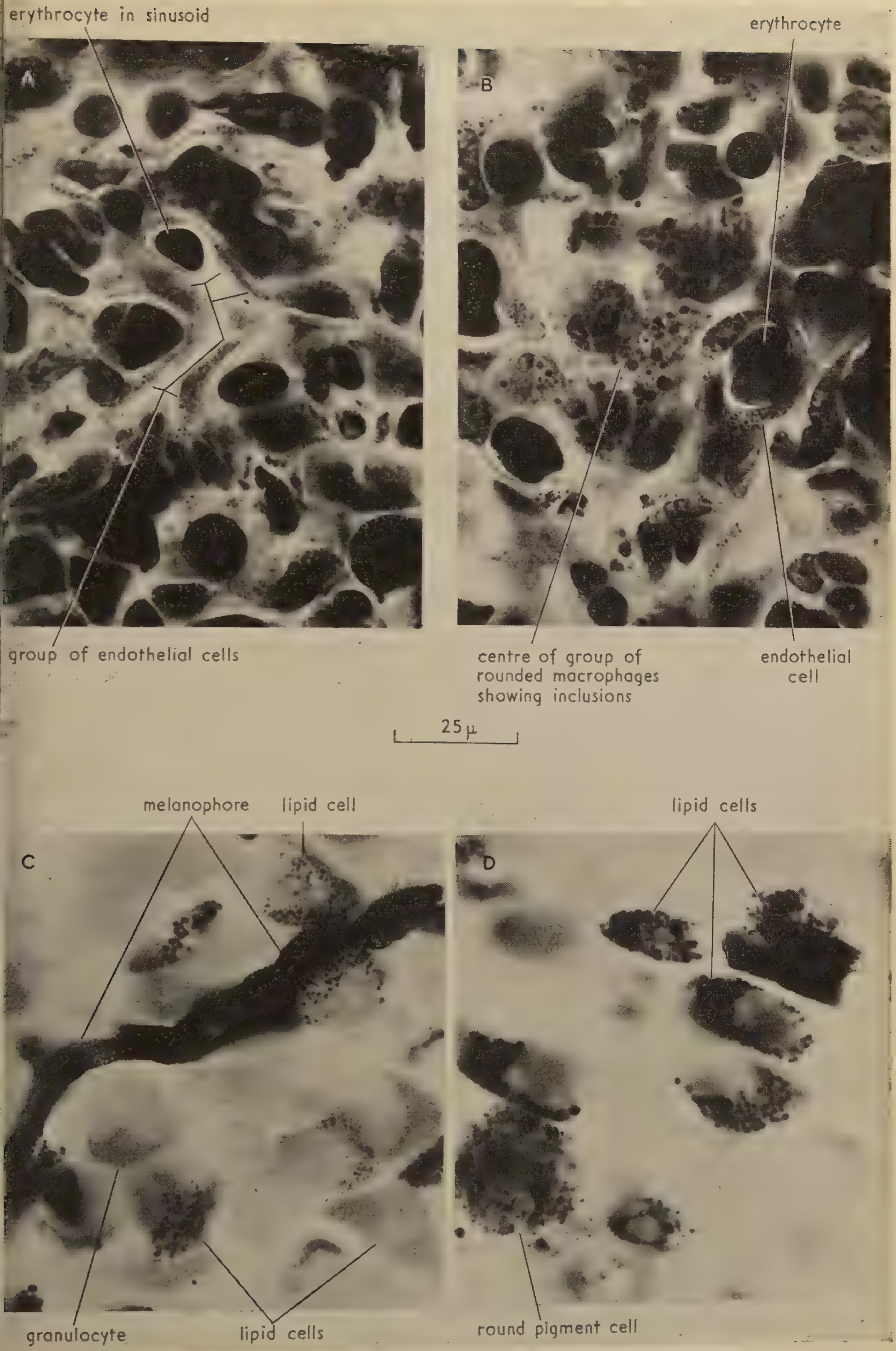

Fig. 5

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ake up a very faint grey coloration with Sudan black, and also give a faint positive reaction to the Ashbel–Seligman test. However, they give none of the characteristic reactions of carotenoids; thus their colour is not affected by concentrated sulphuric acid, nor by a solution of antimony chloride in anhydrous chloroform; they do not react with iodine in potassium iodide, according to Lison's procedure (1953), and they have no autofluorescence.

The remaining class of coloured compounds to which the non-melanic inclusions of the round cells may belong is that of chromo-lipids (lipo-pigments). Pearse (1954) has shown in diagrammatic form how these substances change in their reactions to histochemical tests according to the degree of oxidation of the lipid from which they are derived. We do not feel able to press this analysis very far, since the colour of the inclusions is a pale one, the Sudan-colouring faint, Schmorl's reaction negative, and autofluorescence absent. However, they are still present in tissues fixed in cold acetone, and are negative to acid haematein though positive to PAS. The sum of the evidence suggests, but does not prove, that the coloration is due to the presence of chromo-lipid and that galactolipids are also present; the cells may therefore contain these substances as well as melanin.

In the smallest of our lungfish dendritic melanophores are found scattered throughout the mesentery, around various cartilaginous skeletal structures, and within the pancreas. But none are yet present in the peri-renal tissue, though the round-type pigment cells are already to be seen there. The round cells already contain brown granules and large colourless globules of the PAS-positive material. The fact that the globules are colourless in sections of small animals, with smaller cells, makes it possible that the coloration in the adult comes rather from the size of the lipid bodies than from the presence of a coloured compound. Invasion of the tissue by dendritic melanophores very soon takes place, and in animals 70 mm and 75 mm in total length these cells are to be found in their adult position. The evidence of a difference in development reinforces the conclusion that a clear distinction should be made between pigment cells of the two types.

The round-cell nodules. The fourth type of tissue is one which it is at first sight tempting to call 'lymphoid'. It forms nodules, spaced at intervals in the peri-renal tissue so that, unlike the other tissues we have described, it is not continuous along the length of the kidney. The majority of its cells are rounded and with little or no recognizable cytoplasm (fig. 6, A). However, we have made comparative preparations of lymph nodes from the walls of the alimentary canal, and feel that there is sufficient difference to make it desirable to avoid the use of the word 'lymphoid', which has so many implications. Thus in figs. 6 A and 6 B, fixed, sectioned, and stained together, by Linder's method, it will be seen that the peri-renal nodules and lymphoid tissue are not identical in appearance, particularly in the almost complete absence of reticulin fibres in the former. We shall therefore provisionally call the tissue in question 'the round-cell nodules'. They lie adjacent to the sinusoid area, and immediately under the lipid tissue, at the outer edge of the kidney complex (fig. 1).

Mitoses are very rare in this tissue and there is no evidence of cellul differentiation within the nodules: there are only minor, and unsystemati changes in nuclear shape and chromatin pattern. At its edge each node is i contact with the lipid tissue; in many sections one sees appearances such a those in fig. 8, A. Here the node occupies the lower right-hand segment of th

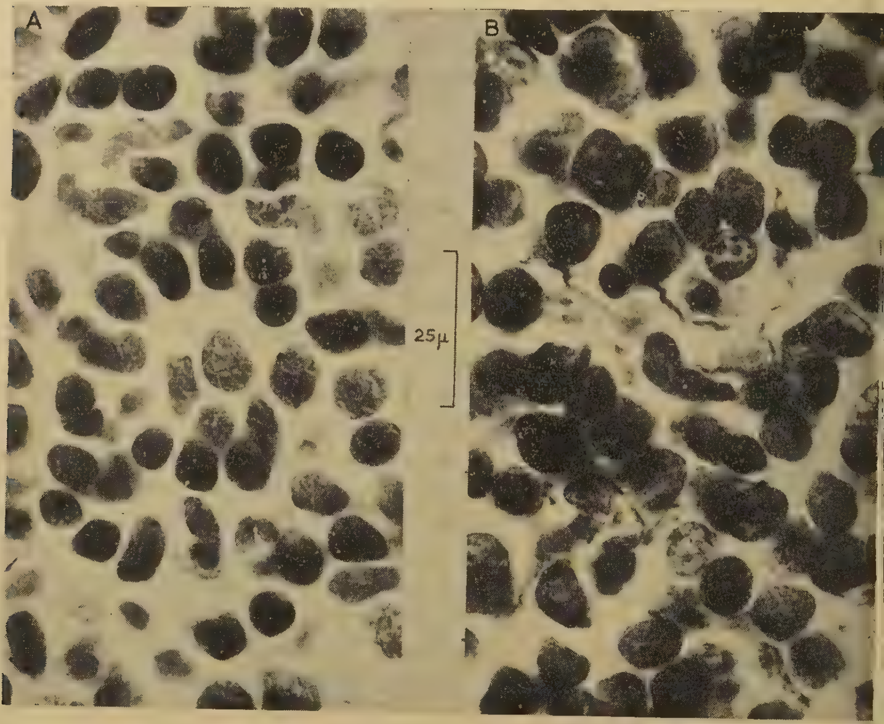

FIG. 6. A, section through a round-cell nodule. B, section through a lymphoid nodule from the gut wall. Both treated identically by Linder's method for reticulin fibres.

photograph. On the lower left-hand side, and passing round and above it, is the lipid tissue. It will be seen that one cell, next to the labelled lipid cell, is very similar in appearance to the cell labelled 'cell with increasing cytoplasm', which is anatomically within the round-cell node. Other similar cells are adjacent, and it may be that the nodes are formative areas for lipid cells. Sometimes these 'cells with increasing cytoplasm' contain a few large lipid droplets; we do not consider, however, that the evidence is sufficiently strong for a categorical statement. It can only be said that the nodes *may* be a germinal centre for lipid cell formation. The possibility that they are a focus of erythropoiesis cannot be excluded, but is an unlikely one.

The steroid tissue. The lipid tissue, pigment cells, and round cells do not colour with Sudan black. The tissue which we are now considering stands out most clearly in Sudan-coloured sections of the peri-renal tissue, for its cells are packed with sudanophil material. These sudanophil cells lie in an area which is topographically constant: they are found at every level, close to the renal vein (cardinal or caval) and along the walls of its factors over a distance of 2 or 3 mm from the point at which they discharge into the vein. They are thus situated ventro-medially, and are intimate not only with the vein but also with kidney tissue within and peri-renal tissue without (fig. 7).

The 2–4 dinitrophenylhydrazine technique and that of Ashbel and Seligman, both used histochemically for the study of steroid substances (with all the well-known provisos), pick out these cells with equal clarity against an unstained background. This is our justification for the convenient name 'steroid tissue' for distinguishing this element. It is similarly the case in the mammalian adrenal that Sudan colouring reveals the same cytoplasmic inclusions as are demonstrated by the techniques in the 'steroid repertoire'.

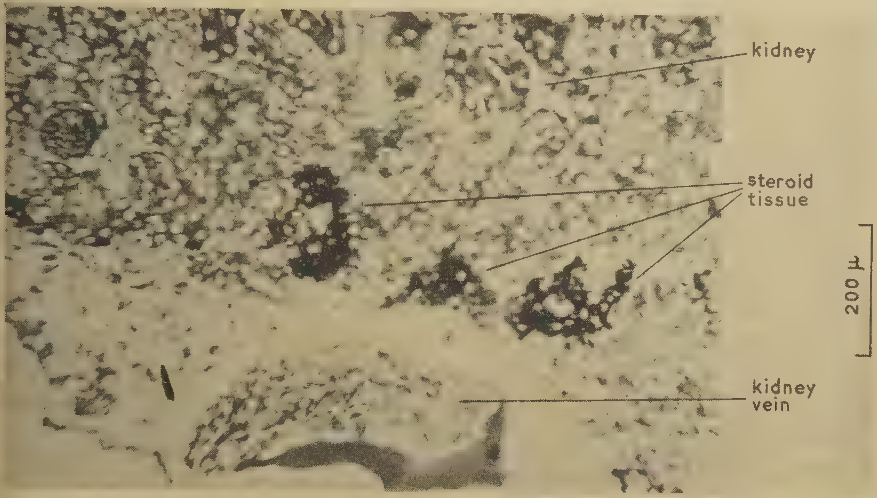

FIG. 7. Transverse section showing the position of the steroid cells. Sudan black.

The structure of the inclusions themselves is not at all easy to distinguish, because of their density in the cytoplasm, and because frozen sections only can be employed to study them. It is best seen by looking through that area of the cell which includes the nucleus; then the layer of cytoplasm is relatively thin, and it can be seen that many, at least, are in the form of hollow spheres (fig. 8, C and D). That is to say that the Sudan or other stain marks out the walls of a sphere whose contents are unstained. Sometimes the wall is thicker at one point than another, or a part of it may be quite unstained. The variety of appearances is strikingly like that figured by Cain and Harrison (1950), in their description of the spheroid complexes and discharge bodies of the mammalian adrenal cortex.

The steroid tissue is far from obvious in paraffin sections, but to an observer who knows where to look it is identifiable by the characteristic pattern of vacuoles left after the loss of lipid; these vacuoles are different in size and arrangement from those in the lipid tissue. Furthermore, the shape of the cells and the ratio of size of nucleus to cytoplasm are characteristic. It is easy to distinguish the steroid cells from other peri-renal elements on these criteria, but very difficult indeed to distinguish them from small groups of renal tubule cells, in paraffin sections. However, when such sections are stained with the azure II / eosin mixture, after Helly fixation, the steroid cells are distinguishable by a pale blue coloration taken up by their cytoplasm.

These cells are the only ones in the peri-renal tissue in which we have regularly demonstrated structures which are indubitably mitochondria. They are feebly demonstrated by acid haematein, which fails to stain the sudanophil material; they are short rods, or spheres, best seen after mitochondrial staining. One feels no difficulty, with these cells, in distinguishing between mitochondria and other lipid inclusions: the size, structure, and staining reactions are so different.

The presence of these steroid cells was first reported briefly by Holmes (1950), who used the Schultz–Liebermann–Burckhardt test. Gérard (1951) repeated this test on sections of *Protopterus dolloi* and showed that, in this animal, the cells are localized in a definite relationship to the renal veins. We have, however, made some observations which suggest that in animals subject to 'stress' sudanophil spheroids appear in cells located elsewhere in the peri-renal tissue (Moorhouse, 1956); furthermore, in a specimen of *P. annectens* similar sudanophil inclusions are found in scattered cells in the lipid tissue. Gérard's conclusions are therefore true of the normal *P. dolloi* and *P. aethiopicus* but do not rule out the possibility of interspecific differences, nor of variations during the life of the individual.

Steroid cells are present in every section which is cut through the kidney, but they are absent from that part of the peri-renal tissue which extends in the body-cavity as a cap on the anterior tip of the kidney. In this they differ from all other peri-renal tissue elements, and it is interesting that their general appearance is similar to that of renal tubule cells. In our youngest fish the steroid cells are already present, in their adult position and with their adult cytological characteristics.

We must record here an unsuccessful attempt to extract steroid substances from the peri-renal tissue; it was hoped that they might be identified by chromatography. There is no conceivable way of separating the steroid cells from the rest of the tissue in fresh material; consequently the only possible approach seemed to be that of extracting the whole kidney and its covering.

Fig. 8 (plate). A, transverse section through the edge of a round-cell nodule. Metzner's method. The cells at the edge of the nodule, between it and the lipid tissue, have an increasing cytoplasm without inclusions.

B, spleen, impression preparation, Leishman. Three kinds of granulocyte are seen; many of the other cells are probably erythroblasts.

C, steroid cells, Sudan black.

D, steroid cells, Sudan IV.

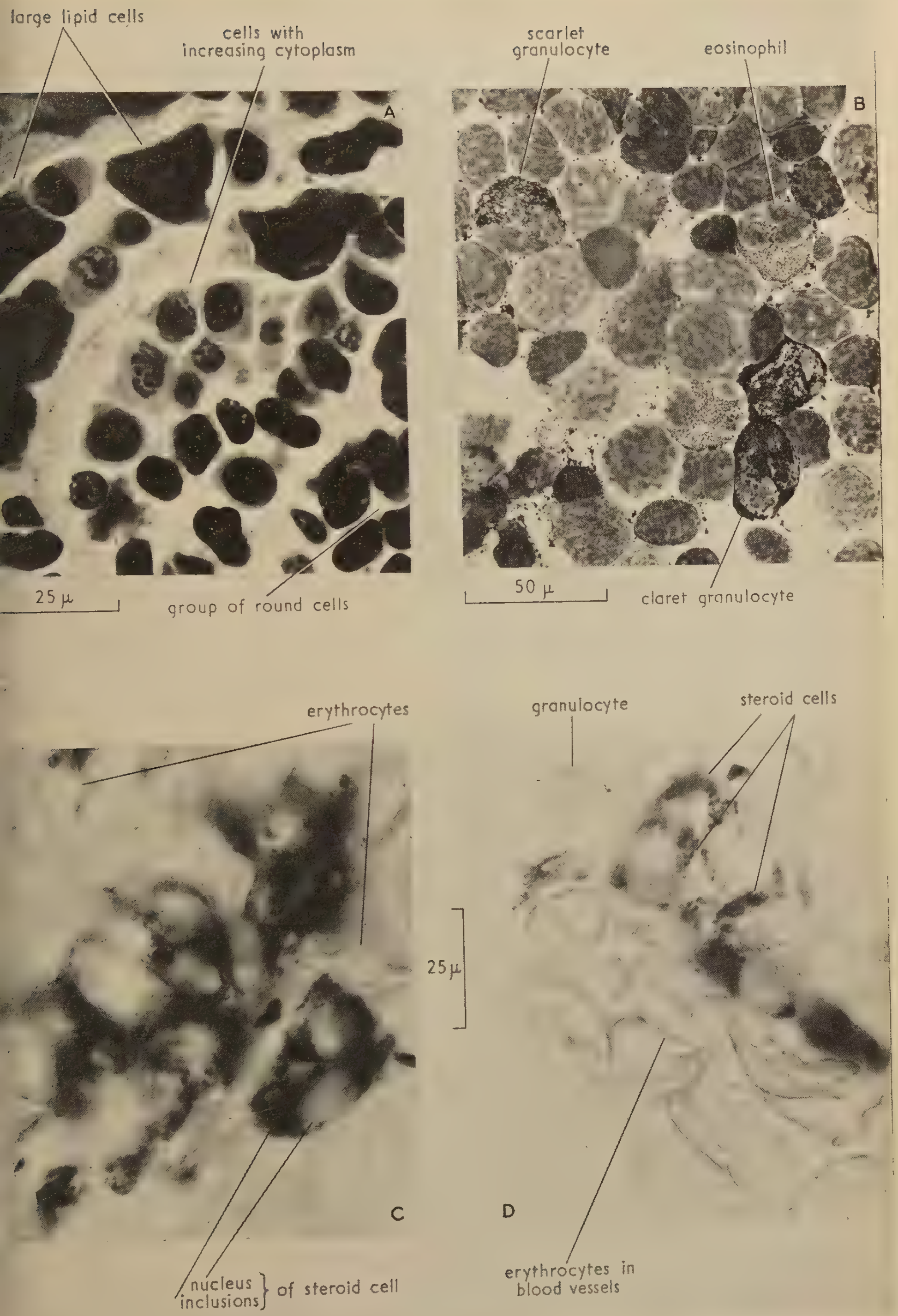

FIG. 8

W. HOLMES *and* D. E. MOORHOUSE

One of us (D. E. M.) made a special journey to Lake George in order to btain very large fresh lungfish.

Six animals, each of a total length of between 1·0 and 1·2 m, were killed by ecapitation; the kidney was immediately dissected out, and chopped up in cetone. This mixture was transported back to Jinja, ground up with sand, ltered, and concentrated by evaporation. Unfortunately at no stage could the olvent or mixture be kept properly cool.

This work was, inevitably, crudely done; nevertheless, Dr. Bush of the National Institute for Medical Research kindly consented to examine our xtract, which was flown back to England. Chromatographic analysis failed to etect the presence of steroid substances.

The identification of myeloid tissue

The problem of the way in which red and white blood corpuscles are ormed in the lungfish has attracted the attention of many haematologists, and ordan (1938) spoke of the spleen of *Protopterus* as being 'perhaps the most mportant tissue in comparative haematology'.

All haematologists who have written on this subject in recent years have held that the peri-renal tissue has a role in haematopoiesis. Since the lungfish s a representative of the ancestral tetrapod line it has been studied in the earch for a primitive generalized type of this process, and when studied by workers with particular theories of blood-cell formation has usually produced confirmation of their views.

Thus Jordan and Speidel (1931) were adherents of the view that the stem ell of all blood corpuscles is indistinguishable from a lymphocyte. Although his view is not now widely accepted it has to be borne in mind when reading hese authors' work, which is an important contribution to knowledge of this ish from two biologists of wide experience.

The lymphoid haemoblast of Jordan and Speidel contains cytoplasmic ranules and its characters are not sufficiently defined, it seems to us, to disinguish it from cells which we consider to be stages in the formation of granuocytes alone. We have seen the later stages in the formation of red blood orpuscles as they describe them; having lost their cytoplasmic inclusions (if hey ever had them) these cells are seen to develop into the adult, nucleated, orpuscles while in the circulating blood (fig. 11, B).

Jordan and Speidel distinguished three kinds of eosinophil; two are related y a series of intermediate forms, so that all stages can be found between a cell with round nucleus and fine granules and one with a polymorph nucleus and oarse granules; the granules are at first spherical, later ellipsoidal. The third ype has a polymorph nucleus and fine granules.

A few years after the publication of Jordan's and Speidel's work Dustin 1934) made a further study of this problem. He, like Jordan and Speidel, ave the name 'special eosinophil' to those circulating granulocytes which ontain eosinophil granules and have a polymorph nucleus. He considered the rue eosinophils to be those which have a lobed nucleus and smaller granules.

Champy and Louvel, however, writing in 1939, and apparently without know ledge of Dustin's work, regarded the leucocyte with eosinophil granulations a neutrophil; that is to say that although they admitted that the granulatio were eosinophil they considered that the cell was homologous with the neutro phil leucocytes of mammals, and justified this paradox particularly on th ground of nuclear shape. They describe the nucleus of their 'neutrophil' polymorph, its granules wine-red or eosinophil, and the intergranular cyto plasm blue.

Jordan and Speidel considered that granular leucocytes are formed not onl in the spleen but also in the peri-renal tissue and in the sub-mucosa of the in testine, along its length from the level of the spleen to the rectum. Champ and Louvel, however, took a different view; they stated that the peri-ren tissue is principally the site of red-blood cell formation, and they interprete the majority of the cells with eosinophil inclusions as a series of stages fro myelocyte to haematoblast, which retained their granulations throughout th earlier stages.

Champy and Louvel's account not only of the blood but also of the blood forming organs is extremely detailed; they mention, in connexion with th peri-renal tissue, many cell types which correspond with those which we hav found. Thus, for example, their polyhedric mucoid cells seem to be the sam as our large cells—though these, in our hands, have given negative results t histochemical tests for mucoid substances. It is unfortunate that Champy an Louvel make no reference to the work of Dustin, for it is difficult to correlat their elaborate study with his work, which succeeded and extended that c Jordan and Speidel. We have, in fact, found no evidence that any author ha as yet made an attempt to reconcile the divergent opinions which haema tologists have expressed about blood-cell formation in the lungfish, in spit of the importance attributed to this problem.

It is interesting to find that though Dustin described his work as a study c the haematopoietic system he was so impressed by the evidence of secretor activity in the peri-renal tissue, particularly in the cells which he name 'cyanophil' and 'orangeophil', and of the presence of secreted droplets in th intercellular spaces, that he came to the conclusion that a part of the functio of the tissue is as an endocrine organ.

A substantial part of our work in studying the histology of the peri-rena system has been to determine whether it does play a part in haematopoiesi and to find out which of the cells we have distinguished can be considered a blood cells or their precursors.

In the absence of an agreed account of the blood-forming process we wer obliged to re-examine this problem, in so far as it particularly concerned u We needed, first, a knowledge of the corpuscles in circulating blood and secondly, a knowledge of the stages of formation of these cells as seen in th spleen. In order to obtain this information blood smears were made; bloo extravasated from the cut tail or taken with a pipette from the great vessel close to the heart was smeared in the usual manner. The smears were fixe ariously by drying, or with the vapour of formaldehyde or that of osmium etroxide. Air-drying gave us the results we liked best, and of the Romanowsky stains Leishman's was chosen for most material, giving the best range f colour, the stain being freshly prepared from powder (B.D.H.).

Since histochemical methods were an important part of our histological work we applied Sudan black and the acid haematein test to the smears, in the way described by Bloom and Wislocki (1950) for their study of mammalian blood and bone marrow.

The cells found in the spleen were studied by an impression technique, and n teased portions of fresh tissue. To make the impressions a fresh specimen of the spleen was cut, and the cut surface gently but firmly applied to the urface of the slide. The impressions and teased preparations were treated as f they were smears, that is to say, some were stained with Leishman's stain, others with Sudan black or acid haematein. The impression preparations of he spleen contained an abundance of cells such as is seen in fig. 8, B. Here there are three distinct types of granule cell, and preparations of this kind would no doubt be most informative to a haematologist.

It would have been of the greatest assistance in our work if comparable impressions could have been obtained from the peri-renal tissue so that we could assess its status as a haematopoietic organ by comparison with the spleen, of which the function is beyond doubt. However, in spite of repeated attempts we never obtained satisfactory impressions of the peri-renal tissue; few or none of its cells detached themselves when the same procedure was followed as was successful with the spleen. We feel sure that this reveals a real difference between the spleen and the peri-renal tissue: in the latter very few cells are loosely held in the connective reticulum and, therefore, relatively few are about to be liberated into the blood stream. In fact almost all the cells in our impressions were those of normal extravasated blood.

The published accounts of lungfish blood are based sometimes on smears, sometimes on sections stained with Romanowsky mixtures, and it is not always clear which type of material was the basis of the descriptions. The differences between leucocytes in smears and the same cells as seen in sections of blood-vessels are considerable, and at first caused us much perplexity. The Romanowsky colour-reactions are, of course, different in the two cases, and there is a marked difference in the sizes of the cells, and in the shape of the inclusions. It is necessary to describe some observations on these differences, for they are important in the understanding of our illustrations and may ultimately help to resolve some contradictions in the literature.

We find that the most common granulocytes have a mean diameter of close to 30μ in blood smears and of from 14 to 17μ in sections (erythrocytes have a length of about 45μ in smears and from 26 to 30μ in sections). Furthermore the cytoplasmic granules are variously round, ovoid, or ellipsoidal in smears, but in granulocytes seen in gelatin sections of blood-vessels they always appear almost perfectly spherical (compare fig. 9, A, B, C, and D). Another point is that these lungfish cells are so large that they are very commonly cut up when paraffin sections are prepared at 8μ. Cells with a polymorph nucleus seem t be more common in smears and thick gelatine sections than they are in paraffi sections, presumably because when the cell is cut in pieces by sectioning onl

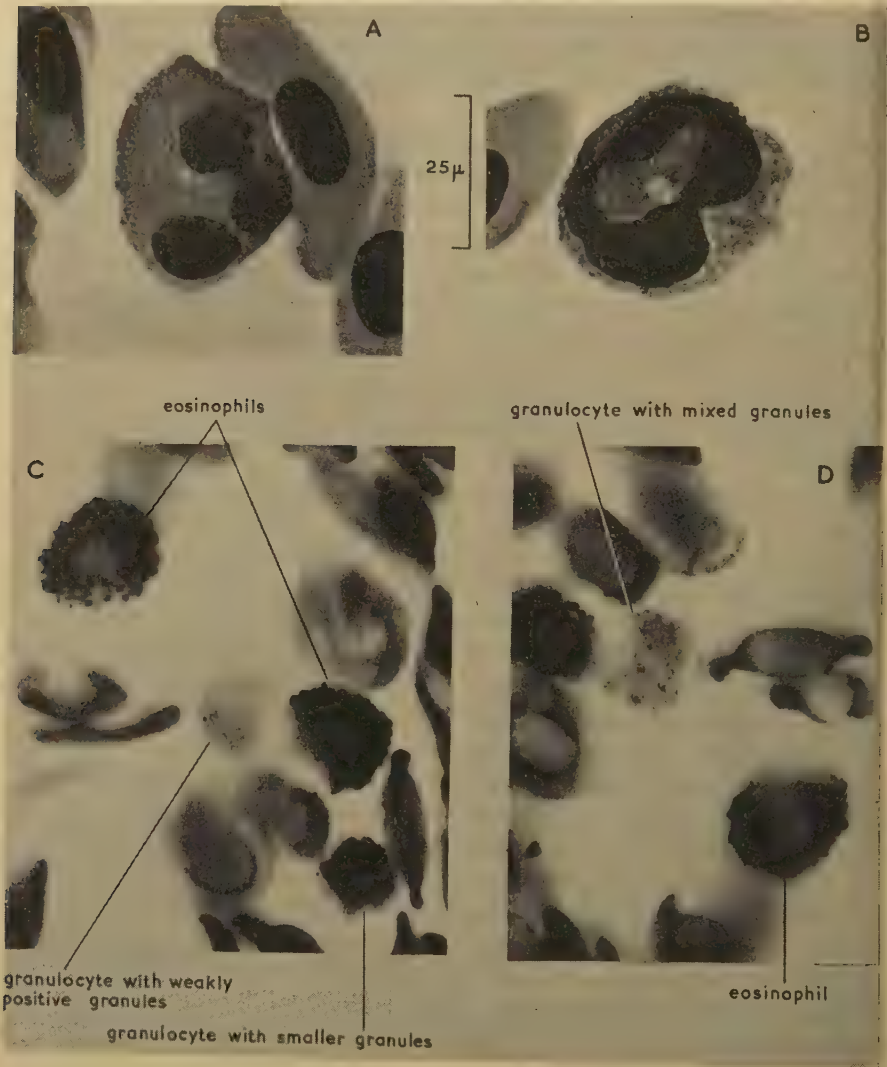
A
B
25μ
eosinophils
granulocyte with mixed granules
C
D
granulocyte with weakly positive granules
granulocyte with smaller granules
eosinophil

FIG. 9. A and B, eosinophils, blood smears, Leishman; much of the intergranular cytoplasm is pale blue. C and D, granulocytes in a blood-vessel, gelatin section, acid haematein.

one lobule of the nucleus may be present in the section, even though it is multilobed. Then the leucocyte would be described as having a non-lobulated nucleus. It is the large size of the dipnoan cells that results in these special difficulties in the study of haematopoiesis.

Ideally the connexion, if any, between circulating granulocytes and the cells of the peri-renal tissue would be revealed by the application of a Romanowsky stain to impressions and sections from the latter. But just as our attempts to make impression preparations failed, so the sections stained with Romanowsky dyes by the various methods listed by Pickles (1950) gave preparations in which the colour spectrum was so different from that obtained with blood smears as to make comparisons dangerous.

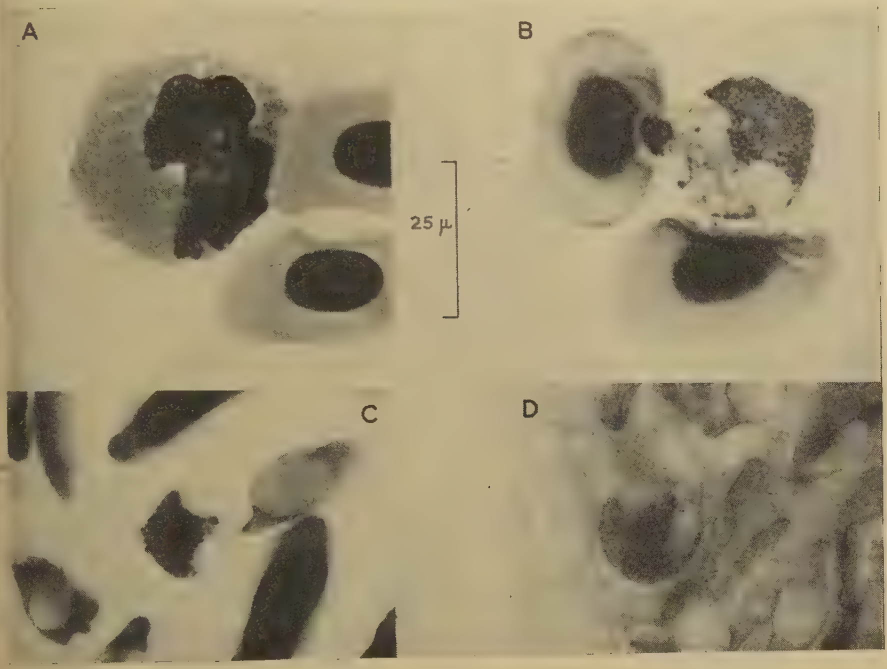

FIG. 10. A, eosinophil, blood smear, Leishman. B, eosinophil, blood smear, acid haematein; the edges of the cell are damaged where they are in contact with the erythrocytes. C, granulocyte, presumably eosinophil, gelatin section, acid haematein. D, granulocyte, presumably eosinophil, gelatin section, Sudan black.

It is the fact that so many of the peri-renal cells are loaded with granules which stain with eosin that has coloured earlier interpretations of this tissue; they stain equally with Lendrum's carbol-chromotrope mixture, which is regarded as a precise stain for eosinophils. The study of sections, therefore, gives little help in the analysis of haematopoietic activity, and we were obliged to depend much more on the results coming from the use of Sudan black and the acid haematein methods which, having validity as histochemical procedures, are more likely to reveal something of the significant connexions between the living cells.

The most common granulocyte in blood smears we shall call the eosinophil. It is illustrated in figs 9, A and B; 10, A. Its nucleus is occasionally merely indented, usually markedly lobulated, and sometimes forms a ring. Its cytoplasmic granules are densely packed and stain an orange-yellow in Leishman preparations; they are sometimes round, more usually ellipsoidal or ovoid. The intergranular cytoplasm shows a distinct pale-blue colour, though not always uniformly so. The dimensions of these cells and inclusions vary, as has been said, according to the nature of the preparation, and can be judged from the photograph. Eosin and carbol / chromotrope stain them intensely in smears and sections.

The reactions of the eosinophil granules to lipid stains are as follows. In both smears and sections the coloration with Sudan black is either feeble (fig. 10, D) or absent. The typical reaction of the eosinophil to acid haematein, in a blood smear, is for the granules to be strongly positive; each is surrounded by an unstained halo and the rest of the intergranular cytoplasm is grey (fig. 10, B). In sections stained with acid haematein the eosinophils most commonly show large, strongly positive, spherical inclusions (fig. 9, C and D). Sometimes, however, the inclusions, while reacting similarly, are smaller, and the cell as a whole smaller (figs. 9, C, 10, C). It seems therefore that some growth takes place amongst circulating eosinophils.

Other kinds of granulocyte are seen in Leishman smears, though they are much less common than eosinophils. Two of the more common are illustrated here (fig. 11, A and D). Their colour in such preparations is described in the legend to the figure. Their occurrence has to be borne in mind when interpreting certain cells seen in acid haematein preparations. Thus in fig. 9, C there is one granulocyte whose granules are very feebly stained; in figs. 9, D and 11, C are cells containing smaller granules than those of the 'normal' eosinophil, some much more deeply stained than others. Such forms may either be stages in the development of eosinophils, or granulocytes of another type.

Dynamics of the peri-renal tissue

The description of the parts of this tissue has shown that though the whole has the unity of an organ it is regionally differentiated within itself into zones with distinct histological characteristics. We have now to consider how far our observations of the cytology and cytochemistry of the parts can be interpreted in terms of function, while appreciating that conclusions based on the microscopic investigation of fixed material can only be provisional.

The steroid zone seems to present the least difficulty: in both location and cytology it is the most clearly defined element. Its cells show no difference in structure between any of the normal animals we have studied except in details of the form of the sudanophil spheroids. It is composed of relatively few cells; these are not all contiguous with each other but they are confined to a definite location determined by the anatomical distribution of the main venous trunk of the kidney and lie in relation to this trunk and its proximate factors. The steroid cells are in small islands, very close to the endothelium of the vein. The connexion between these cells, the kidney, and its venous drainage is demonstrated by the absence of the steroid cells from the anterior cap of peri-renal tissue. We have found no evidence, even in our smallest animals, that these cells are growing, dividing, or being replaced by the immigration of parent cells from the adjoining tissue. Nevertheless, the intimacy between the steroid cell islands and the other peri-renal cells which separate them speaks for some functional connexion.

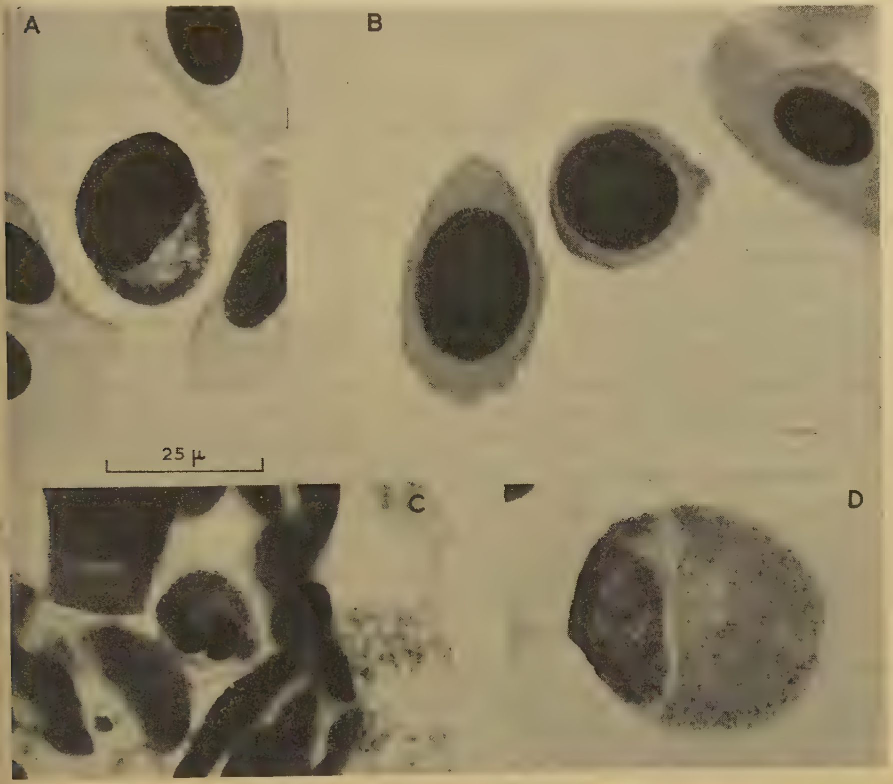

FIG. 11. A, granulocyte, blood smear, Leishman; granules mixed red and blue at the edge of the cytoplasm, all red within it. B, three stages of the intravascular differentiation of the erythrocyte; blood smear, Leishman. In the first stage the nucleus is round and purple; in the other two it elongates and becomes blue and smaller, while the extra-nuclear substance increases. C, granulocyte, paraffin section, Metzner's method. D, granulocyte, blood smear, Leishman; granules orange-red, a blue band between granular zone and nucleus.

We are not able to add anything to discussions of the chemical significance of a positive reaction to the 'steroid repertoire' (see Deane and Seligman, 1953). In histochemistry our steroid cells correspond with those in the mammalian adrenal which are positive to Sudan, negative to acid haematein. The presence of spheroids of varying size is strongly indicative of secretory activity of an endocrine type.

The lipid tissue is readily identified in most kinds of histological preparation by the abundance of intracellular acidophil droplets. The arrangement of its cells may be regular and fasciculate, when rows of cells lie parallel with each other, separated by a fine connective reticulum, or more irregular and reminiscent of the organization of the reticulate zone of the mammalian adrenal. There are great differences between individual animals in the relative amounts of lipid tissue organized in the fasciculate and reticular ways. We believe that all these variations are likely to be significant, but we have failed entirely to establish any correlation between them and variations such as the age or sex of the animal, or the season of the year, or any other differences that it was in our power to measure.

The same kinds of cell are always found in the lipid tissue, but the proportions of the kinds varies, and this, too, must be significant. Because of the continuous variation between cell and cell in the number and size of the characteristic cytoplasmic droplets it is impossible to give a straightforward account of the structure of a limited number of kinds of cell; we are forced to the conclusion that any one preparation shows a cell population of which the diversity arises from vital activity of the tissue, and manifests itself most obviously in the production, growth, and discharge or dissolution of cytoplasmic inclusions. Our account must therefore be framed in terms of a provisional hypothesis of the sequences in cell differentiation and activity which result in the apparent heterogeneity seen in a single microscopical preparation.

In the lipid tissue cells characterized by a rounded nucleus, little cytoplasm and no distinct cytoplasmic inclusions may be regarded as the parental type, though they are not necessarily the youngest cells, for they may have reverted to a presecretory phase after loss of their inclusions (figs. 3, D, 8, A). These cells are often the most numerous in the tissue, though they are the least obvious, for they lack the distinctive inclusions of other forms. The cytoplasm gives a positive reaction to the periodic acid / Schiff procedure, and has a slightly granular appearance. These parental cells appear to give rise to the lipid cells with large inclusions, which we shall call 'large lipid cells', in the following manner: the nucleus increases somewhat in size but remains spherical; the cytoplasm extends and as it does so cytoplasmic droplets appear and become progressively more numerous. As soon as the first droplets are recognizable the cytoplasm has ceased to give a positive PAS reaction. The droplets are first seen in acid haematein preparations as spheres homogeneously coloured grey. When grown to their maximum size the cells are either cubical or rounded, according to whether the organization of the tissue is fasciculate or reticular, and the droplets stain homogeneously fully black with acid haematein. At no stage do they colour with Sudan black. They are so numerous as to pack the cytoplasm (fig. 3, A and B) and are sometimes so compressed as to assume a flat-sided, roughly pentagonal shape. It is difficult to see whether mitochondria lie between the inclusions, for the latter colour intensely with mitochondrial dyes.

There is abundant evidence that the phase of elaboration of full-sized lipid droplets is followed by one in which the contents of the droplets change chemically and are wholly or partly discharged from the cells. Mitochondrial preparations show a number of large lipid cells in which the droplets are negative to the stain, though their position is indicated by unstained 'vacuoles' in the cytoplasm. These are the only lipid cells in which one can distinguish mitochondria, which lie between the unstained droplets. Some such cells may contain both positive and negative spheres intermingled, or smaller positive droplets amongst larger negative 'vacuoles'. The results with acid haematein parallel these and the 'empty vacuole' appearance is commonly seen (see the illustrations in Holmes, 1950). It is tempting to believe that the droplets are discharged entire from the cells, for it is a common appearance to find groups of haematein-positive droplets lying about in the tissue spaces. One has to be cautious, however, with such appearances, for it is not easy to be sure that such aggregations of droplets are not still within a tenuous cytoplasmic veil. It is probable that some material remains in the vacuoles when they cease to react with acid haematein. For in Azan preparations, in which the lipid inclusions colour bright red both within the cells and when presenting the appearance of discharge, the empty vacuoles colour a pale pink, purple, or yellow, distinct from the orange cytoplasm.

These observations we think to be indicative of endocrine secretory activity by the lipid cells with large inclusions. We believe that these cells cannot give rise to granular leucocytes for the principal reason that their inclusions rapidly grow to a larger size than those of the leucocytes. Further, the nucleus never shows any sign of departure from the spherical shape.

The lipid cells with small cytoplasmic granules—small lipid cells—are now to be considered; the granulation is sharply demonstrated by acid dyes and particularly well by Lendrum's carbol-chromotrope. The sizes of these cells and their contents can be seen in fig. 3, C. In Azan preparations the inclusions are red with a purple tinge, and are thus distinct from the cells with large inclusions, in which they stain bright red. The cells are found in two forms, principally distinguished by the shape of the nucleus, and there are strong indications that the second type is formed from the first. The first type is the cell which, of all the components of the peri-renal tissue, is the one most commonly seen in mitosis (fig. 3, C). Its nucleus is round, and the cell as a whole is rounded. The cytoplasmic inclusions are negative to Sudan black and give only a brown coloration with acid haematein; they colour intensely with mitochondrial dyes. The second type is characterized by having a nucleus which is either notched or symmetrically bilobed, the waist between the two lobes being broad. The inclusions are often slightly larger than those of the first type; they are faintly sudanophil, and colour grey-black with acid haematein (figs. 3, C, 5, C).

Salazar's protein test proves to be a useful method of distinguishing this second type of small lipid cell, though the histochemical significance of this finding is obscure. The nuclei and inclusions of the large lipid cells are negative to the test. The small lipid cell with lobed nucleus, on the other hand, stands out sharply in such preparations (fig. 4). The nucleus colours darkly, and the closely packed inclusions appear as spheres surrounded by positively reacting material; it is impossible to say whether the positive reaction is given by the intergranular cytoplasm or by the surface of the granules. This material gives good evidence that the cell with a round nucleus can develop into one with a lobed nucleus, for a series of forms can be distinguished in which, while the granules are developing their positive reaction, the nucleus is changing shape.

The outstanding questions about these small lipid cells are: from what parent cells are they formed, and do they ultimately become fully polymorph circulating granulocytes? On the first question we are still uncertain in spite of every effort to decide. The simplest theory is that they arise from the same parent cells as the large lipid cells, being differentiated at an early stage by formation of smaller and more numerous inclusions. But we cannot find enough intermediate forms to satisfy ourselves that this is certainly so, but feel that it is the most likely possibility. We are more certain of the answer to the second question, and believe that the cells with a bilobed nucleus can become fully polymorph granulocytes. The bilobed cells are commonly seen in the blood vessels of the kidney and peri-renal tissue, and correspond with the granulocytes with fine haematein-positive granules. Appearances suggest that the transformation of the cell with bilobed nucleus to the eosinophil as seen in blood smears takes place within the vessels and is accompanied by growth of the inclusions and a chemical change in the inclusions which progressively increases the positive reaction to the acid haematein test.

This is not to say that we believe that the peri-renal tissue is an organ of granulopoiesis of the same status as the spleen. Appearances in sections and impressions of the spleen are so different from those in the peri-renal tissue: in the spleen mitoses are abundant, impressions take off large numbers of free granulocytes, and many stages of granulopoiesis as well as of erythropoiesis can be demonstrated with ease (fig. 8, B). It is probable that the physiological significance of these small lipid cells is more subtle than is implied by the simple statement that they form a granulopoietic tissue, for they must be involved in the lipid metabolism of the whole peri-renal organ.

The phagocytosis of injected trypan blue proves that the endothelial tissue forms a macrophage system. Its cytology shows characteristics common with the cells of the reticulo-endothelial system of higher animals, particularly in the presence of cytoplasmic inclusions which are stained by mitochondrial techniques, but which are somewhat larger than the more usual forms of mitochondrion, and show some staining reactions which are unusual for these bodies (p. 133).

It has been shown that macrophages of this system can exist in a rounded form, and there is strong evidence that the pigment cells which have the same shape are derived from cells of this type. Some of the endothelial macrophages contain a few small melanin granules which reduce ammoniacal silver; when silver impregnation is followed by reduction in a mixture containing hydroquinone, most of the endothelial cells are found to contain fine black granules.

The round pigment cells form a series in the number and size of the melanin granules they contain, and the endothelial cells can reasonably be taken to represent the first stages in this series. The fact that the round pigment cells lie either within the endothelial zone or at the lower border of the lipid tissue which adjoins it suggests that it is a function of the macrophages to take up or elaborate melanin. The globules of iron occasionally found in these cells indicate that they may also be concerned in the destruction of the contents of aged red blood corpuscles.

Jordan and Speidel noticed macrophages in the peri-renal tissue, and stated that they are frequently found to contain the debris of 'eosinophil' cells. In sections stained by the Azan method we have often seen round pigment cells whose appearance convinced us that they contained fragments of broken-down large lipid cells, in the form of inclusions whose shape and staining reactions were similar to those of the 'empty vacuoles' of those large lipid cells which are negative to acid haematein.

The presence of PAS-positive material in round macrophages and pigment cells, in a similar form, is further evidence of the translocation of lipid in this tissue, and the positive PAS reaction of young lipid cells may also be a relevant indication, since it is present at a stage immediately preceding the differentiation of the intracellular lipid droplets.

The problem of the interpretation of the histochemical observations is closely similar to those which have arisen in the study of the mammalian adrenal cortex. Lillie's study of the PAS reaction contains some very relevant comments (1950). He points out that, while the cutaneous melanin of the guinea pig is negative to PAS, the so-called melanin of human melanosis coli gives a positive reaction. He recalls the suggestion by Hueck that the 'brown granular lipofuscin' of the zona reticularis of the adrenal is 'closer to melanin than some other lipofuscins'. The adrenal cortical cells may contain a black melanin and a yellowish-brown lipochrome, often side by side (Bachmann, 1954). Bachmann (1939) has shown that in the adrenal cortex one may trace a series in which cells become progressively more heavily pigmented; from a condition in which cells contain both sudanophil droplets and fine yellow granules to one in which lipid is absent and pigmentation maximal. In the adrenal also the presence of inclusions containing iron is associated with that of melanin and yellow droplets.

We have found no evidence of blood-cell formation in this zone, but cannot deny the possibility that red corpuscles are manufactured there. It seems most unlikely that it produces granulocytes; for though many of these cells are found within it, there are no evidences of their formation there, and they are often clearly within the sinusoids.

Finally, we should mention some methods of investigation we have used, but which have not given useful results. We have not been able to obtain information on the intracellular location of enzymes because the recommended fixative, chilled acetone, proved so destructive to the cells in which we were interested. Its effect on the intracellular droplets, and consequently on the cytoplasm, was such as to produce preparations unsuitable for cytological study.

The method of fixation required for the localization of vitamin C was equally destructive, and the manner in which the silver was deposited in the subsequent processes obviously bore no relation to the distribution of any substance located in the living cells.

Attempts were made to demonstrate the intracellular reticulum of Golgi, in the hope of examining its relation to the liposomes and other inclusions. The method of da Fano was employed, but the results do not justify description since the reticulum showed neither a 'classical' appearance nor any regularity of form in any particular type of cell.

COMPARATIVE HISTOLOGY

Granulocytes

Histological analysis of the tissue we have been describing began more than fifty years ago; the first account which contained cytological detail was that of Drzwina (1905). She spoke of the presence of large polyhedric cells whose cytoplasm contained granulations of varying size, often eosinophil, but sometimes having no reaction to stains; these seem to correspond with our large lipid cells. She also mentioned the presence of 'leucocytes' with a slightly curved or indented nucleus, and cells with 'acidophil granulations' distinct from the polyhedral cells.

Stephan (1906) also noticed the polyhedric cells; he concentrated his attention on the changes in the histology of animals which regularly undergo 'aestivation'. He observed the cells with acidophil-eosinophil granulations and claimed that these are formed, in large numbers, from the polyhedric cells at the moment when the lungfish abandons its torpid state of aestivation. He further concluded that the granules of the eosinophils are from time to time discharged, leaving behind vacuolated cells which correspond with the polyhedric cells with non-staining granules of Drzwina and may correspond with our large lipid cells in some stages of their life history.

The comparative study of the histology of vertebrate animals is easiest, and has prospered most, where it is concerned with organs whose homologies are already established. In the earlier history of lungfish studies only one likely homology was suggested for the peri-renal tissue: that it is a haematopoietic organ, and that it should be compared with the spleen and other haematopoietic foci in related animals.

Thus it is that we owe to haematologists all the more modern accounts of the peri-renal tissue.

Comparative haematology is undoubtedly the most advanced branch of the comparative histology of lower chordates, in so far as this can be measured by the volume of the literature. It would not be appropriate here to attempt to review all that has been written on blood-cell formation in other fish, but we wish to draw attention to some relevant points in one of the most recent contributions. Catton (1951), writing on certain teleost fishes, described the cytoogical characteristics of certain 'coarse granulocytes' which are found 'in arge numbers' in the kidney, and at limiting membranes such as the perioneal epithelium. The granules in these cells 'give a positive result with the mith Dietrich test for lipoid material' and there were appearances suggesting hat the granules were in the process of discharging a fluid at the epithelial urface with which they were in contact. There is a suggestion here of a family elationship with our large lipid cells, and it is to be noted that Catton, looking or similar phenomena in eosinophil granulocytes, records: 'I have been unble, however, to find examples of the discharge pattern phenomena described bove in any mammalian tissue examined'. He refers to Stephan (1906), who lescribed the cyclic changes in granule cells in peri-renal cells of *Protopterus*.

We cannot offer any account of lungfish haematogenesis which will reconcile the contradictions in the literature; it seems clear that any such attempt hould come from a research directed towards that end alone. We believe that ve have seen, in smears and sections, all the kinds of cell that have been numerated by other workers. But when so much has been made to turn on differential colour reactions with diverse mixtures of stains, and when standardized quantitative data are lacking, it will do no good to add yet another set of hypotheses. We certainly do not feel competent to do so. Had we been able o obtain impression preparations from the peri-renal tissue we should have been better able to produce an account based on comparisons of this tissue with the spleen. But the negative observations that cells are not readily detached from around the kidney and that the spleen contains more kinds of granulocyte than the peri-renal tissue are likely to be significant. Quite another difficulty comes from our finding that every lungfish taken from Lake Victoria s host to a number of parasites; these include several kinds of trematode, in he gut, and a sporozoan common in the liver and capable of infecting other viscera. Indeed, we spent a good deal of time in determining that certain common cells in our sections were stages in a protozoan life cycle. If fish are like mammals in their reactions to parasites, none of our animals can be regarded as normal so far as their granulocytes are concerned.

It seems that the most fruitful way in which we can express our conclusions on this matter is as follows. Cells indistinguishable from granular leucocytes are present in considerable numbers in the peri-renal tissue. They are found with polymorph or only bilobed nuclei. Cells which may be the parents of this ineage have round nuclei and cannot be distinguished from other cells which become large lipid cells; these latter are easily distinguishable from granuocytes. So far as our histochemical work has gone it demonstrates much in common between the granulocyte line of development and that of the large ipid cells; at the same time it shows that the adult cells are different. It is suggested that, in these primitive fishes, there is an intimate connexion, through morphogenesis and, particularly, lipid metabolism, between circulating granuocytes and the 'sedentary' cells called 'large lipid cells'.

We have found no evidence that red blood corpuscles are manufactured in he peri-renal tissue.

Brown fat

The cells of brown fat, a kind of adipose tissue found in particular locatio in certain mammals, bear some resemblance to the cells of the lungfish lip tissue. Since brown fat is said to be most extensively developed in anima which hibernate it is of interest to consider whether it may have a significa similarity to a part of the peri-renal tissue. It is found in several sites in ra and mice and some lies around the vessels in the hilum of the kidney (Fawcet 1952). It is 'histogenetically and physiologically distinct from ordinary fat', a Fawcett has shown, and his histochemical analysis makes comparison qui easy. The cells of brown fat contain large numbers of discrete cytoplasm droplets; these are, however, unlike the lipid tissue in being sudanophil an negative to the acid haematein test. The phospholipid is concentrated in ver numerous mitochondria. In the face of this dissimilarity, and of the evolutionary gap between fish and mammal, it would be unprofitable to pursue th comparison farther. But it may well be that, when the physiology of lipid tissu and of brown fat are better understood, comparative histology may help b finding a link between them.

The adrenal cortex

It was suggested by Holmes (1950) that a part of the peri-renal tissue ma be the homologue of the adrenal cortex of mammals. This suggestion wa misunderstood by Gérard (1951), and it must therefore be emphasized agai that it does not necessarily imply that the peri-renal tissue has all or any of th functions of the adrenal cortex as manifested in higher animals. In the development of our knowledge of the mammalian cortex, knowledge of functio preceded histochemical study; in the case of the lungfish it is hoped that th knowledge of structure will stimulate the investigation of function and mak it possible to correlate the two. An investigation of the conditions under whic histochemical change can be induced experimentally in the peri-renal tissu will be reported by Moorhouse (1956).

The lungfish chromaffin cells lie close to the origin of the intercosta branches of the dorsal aorta (Holmes, 1950). The dorsal peri-renal tissue i thus near to the homologue of the tetrapod adrenal medulla.

The steroid tissue, being ventral, is rather far from the medullary homologue, but its location in relation to the kidney veins gives strong support fo the evidence of histochemistry, which suggests that it is the homologue o some part of the cortex. Thus in *Petromyzon* and *Lampetra* the cortical homologue (inter-renal tissue) is separated only by an endothelium from the lume of the main renal veins (Giacomini, 1902; Gaskell, 1912). The peri-renal tissu when first seen is in or immediately below the coelomic epithelium, a positio corresponding with that of the adrenal cortex of all those vertebrates in whic such a tissue has been recognized, and its development studied.

The lipid tissue is more massive than the steroid area; the arrangement o its cells often resembles that of parts of the mammalian cortex, but it would no be convincing to press this analogy too far, for the zonation of the mammalia ortex, though no doubt significant, is perhaps primarily a representation of he organization of its vascular system.

Most histochemical studies of the mammalian cortex are concerned with its ntracellular lipid, and there is no doubt that the differences in the lipid picture' in different zones, and the evidences of cycles of lipid formation, are onnected with the dynamics of cortical function. In the lungfish the appearnces are suggestively similar, though in this animal the cells shown to conain phospholipid never give evidence of steroid content. Perhaps at this early tage in evolution these two aspects of adrenal dynamics were more definitely dissociated. The absence of sudanophilia from the lungfish phospholipid is, n a sense, a minor feature, which can only be interpreted when the fundamental histochemical problem has been explored. Studies of the inter-renals of other fish are usually unhelpful in this context, being rather micro-anaomical than histochemical, though Aboim (1944) has demonstrated three ypes of cell in the Selachian organ, distinguishing them on the size of their lipid inclusions, and has shown the presence of melanin in some of the cells, and a positive reaction to the phenylhydrazine test.

Haematopoietic tissue is found in relatively small amounts in the adrenals of mammals; in *Lampetra*, according to Gaskell (1912), inter-renal tissue is found closely associated with the pronephros, and Holmgren (1950) has shown that the pronephros of another cyclostome is the site of active haematopoiesis. Perhaps the intimacy, in lungfish, between blood-cell formation, adrenal-like tissue, and the kidney may be a clue to the functional association of these tissues in the mammal.

A sinusoidal system and its macrophages are found in the mammalian organ, and have been seen by Dittus in the elasmobranch inter-renal (Dittus, 1940). The evidence from the lungfish is that these cells not only form a vascular system, but also take part in the transfer of material within the peri-renal organ.

The lungfish is not a convenient laboratory animal in regions away from its habitat, so further studies of its physiology may be delayed. But for investigation of the adrenal system of vertebrates between fish and mammal it seems to be established as a primitive type that is better for comparison than the more aberrant elasmobranchs or teleosts.

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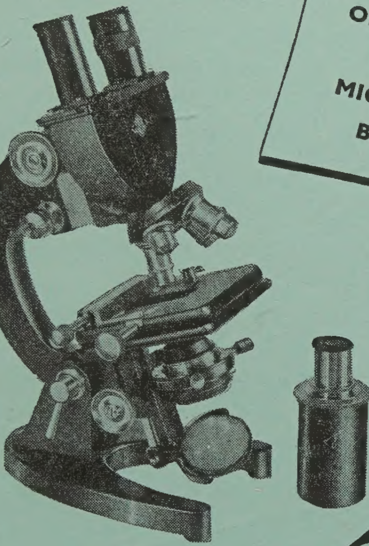

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